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EDITOR'S VIEWPOINT

Hill, Sims, and Newman (p. 448) found small, but not practically significant, reduction in caries activity during a one-year trial of a penicillin-containing dentifrice. This same laboratory had reported failure to reduce caries by use of a penicillin-containing dentifrice, while another worker reported marked caries reduction. Still another laboratory had found no beneficial effect. This, again, emphasizes the importance of studies by several laboratories before any "therapeutic dentifrice" is accepted as being of practical value.

Hill, Rasch, and Wollpert (p. 453) investigated *Streptococcus viridans* and other organisms from the salivas of children using penicillin-containing dentifrices and control dentifrices. They found significant increases in penicillin resistant organisms from the salivas of penicillin dentifrice users, although the other children also had penicillin-resistant bacteria in their salivas.

Kutler and Ireland (p. 458) applied 2 per cent fluoride solution to teeth of young adults and after one year found no significant reduction in new carious surfaces. Since Klinkenberg and Bibby and Rickles and Becks found reduced caries activity in adults after fluoride applications, the differences must be reconciled. (An earlier study used only a single application of 0.5 per cent solution and is not considered for this purpose.) Differences in technic on application or examination do not appear to explain the difference. Since 127 teeth became carious in the 147 test subjects, there may have been a high degree of protection without the topical fluoride. The fact that forty-seven individuals developed no new carious lesions supports this view. More evidence should be required before discarding topical fluoride applications as caries control measures in adults.

Grutsch, Nebergall, Muhler, Fischer, and Day (p. 463) describe a procedure for routine determination of fluorine in waters containing other minerals. Since fluorides are being added to large numbers of water supplies, and since excessive amounts of fluoride may result in mottling, if consumed during tooth development, careful control of the fluoride level is imperative.

Fleming (p. 469) studied the effects of ingested and injected fluorides on transplanted tooth germs. Maturation of both enamel and dentin was slowed in the animals receiving fluorides and the structure of the ameloblast was altered. These, and other changes in transplanted tooth tissue, and on a fast growing transplantable mouse tumor, led the author to the conclusion that fluorides slow down mineralization in transplanted odontogenic tissue and that the action appeared at the enzyme level, but the mechanism of fluorides as caries inhibitors is still an enigma.

Fosdick, Calandra, Blackwell, and Burrill (p. 486) offer another suggestion for a therapeutic dentifrice. They approached the problem by seeking an enzyme inhibitor that would be retained by the dentobacterial plaque. To find such a substance, the authors investigated the acid-inhibiting properties of 381 compounds. From these, ten were found which would attach themselves effectively to the plaque. Three of these were able to keep the pH of the plaque above a cariogenic level and this led to clinical tests on two, sodium lauroyl sarcosinate and sodium dehydroacetate. Unfortunately, clinical studies have not yet been reported that would permit acceptance of dentifrices containing either of these compounds as being "therapeutic." We hope that the studies reportedly in progress will be more encouraging than those from other theoretically "therapeutic" dentifrices.

Dreizen, Reed, Niedermeier, and Spies (p. 497) investigated the salivary flow and potassium and sodium levels of saliva in relationship to its buffering capacity. It had been previously observed that stimulated saliva had a greater buffering capacity than resting saliva. The present study appears to establish that the greater buffering capacity results from an increase in sodium and bicarbonate from the stimulated salivary glands. The sodium and bicarbonate are blood components, as opposed

to the phosphates, proteins and potassium that are chiefly intracellular. It will be interesting to observe whether the bicarbonate and sodium are at higher blood and salivary levels in caries-immune individuals.

Schneyer (p. 504) has investigated the effect of quinine and urethane as inhibitors of salivary amylase activity. He finds that these drugs exert significant effect on salivary amylase only under conditions of temperature and salt concentration which in themselves would induce amylase inactivation of irreversible type.

In the April issue of the JOURNAL an article by Wright and Jenkins appeared (p. 232). This article on ammonia concentration and acid production in saliva showed a statistically significant relationship between acid production on incubation of saliva-glucose mixtures and the ammonium concentration of the saliva. This extremely interesting observation, in view of conflicting reports of other investigators, was commented on by the Editor in an unrelated manner. Actually, the Editor's Viewpoint for another article by Wright and Jenkins should have carried the comment published in April. We apologize for this editorial lapse. The following comment originally published in April applies to the present article on page 511.

Wright and Jenkins (p. 232) found four times as many intact leukocytes in the salivas of caries-free subjects as in caries-active salivas, although adding the disintegrating leukocytes in the salivas of both groups resulted in counts of no significant difference. It is interesting that studies of this type are often made with little description of, and apparently little attention to, the method for determination of caries-active and caries-free groups. In this instance, those in the caries-free group had only one or two fillings each, and no visible decay. (In correcting proof the authors added the information that the caries-free group had no observable caries to mouth mirror and probe.) Bite-wing x-rays might change the grouping of some of these individuals. Some index of present caries susceptibility would be valuable. Differences in methods of determining caries activity groups may account for differences in observations between groups by different authors. We trust that our readers now understand the preceding remarks, prematurely stated in April.

Grubb and Wands (p. 524) tested cetyl pyridinium chloride against thirty-one strains of beta hemolytic streptococci and a C203 strain. All of the organisms were killed after ten to fifteen seconds with 0.025 per cent cetyl pyridinium chloride in water and in 15 per cent alcohol. Ten per cent sterile saliva did not affect the speed of germicidal action in 82 per cent of the tests, accelerating in 8 per cent and reducing in 10 per cent. The value of a fast acting antibacterial agent on the oral mucosa is pointed out.

On the assumption that para-amino salicylate, used in treatment of tuberculosis, might substitute for para-aminobenzoic acid as a stimulant to lactobacillus growth, Maxwell, Dunning, and Newman (p. 528) tested its in vitro effect against *Lactobacillus casei.* The test indicated that para-amino salicylate is not important as a stimulant of lactobacillus' growth, nor of glycolysis.

Chilton and Fertig (p. 530) discuss the use of comparisons of proportions in estimating the required sample size for use in experiments. Failure to observe these fundamental principles may result in loss of time, manpower, experimental subjects, and money. Either too many or too few subjects in an experiment is wasteful. The Office of Technical Service, U. S. Department of Commerce, Washington, D. C., has published Dr. Chilton's "Analysis in Dental Research." This complete work of value to investigators is available from the above address for $3.00.

Black (p. 541) has evaluated Xylocaine and procaine as anesthetic agents, clinically. Clinical investigations of this type are not easy and the need for use of subjective reactions often leads to questioning the results. Black appears to have approached his problem directly and to have evaluated his results scientifically. He found Xylocaine superior to procaine in that it resulted in shorter onset of surgical anesthesia and longer duration, and in that it caused significantly less change in pulse rate. Subjective reaction and anesthetic efficiency were the same for both agents.

Alderisio and Lahr (p. 548) describe the use of strain gauges to measure the muscle forces of the lip, cheek, and tongue. Re-

sults obtained with this technic should be of value to orthodontists and prosthodontists.

Kraus and Furr (p. 554) report the first phase of a study designed to establish specific dental traits as related to genetic patterns. Using the lower first premolar, they have defined seventeen traits. These have been established as heritable, although the data are not given herewith. The teeth have long been of interest to anthropologists and criminologists as means of establishing evolutionary lines and identity. The chief virtue of the teeth in these instances has been their relative indestructibility. The finer study of their details in relationship to genes may afford unusual opportunities to establish racial patterns and interrelationships.

Kreshover and Clough (p. 565) demonstrated disturbances in amelogenesis in the continuously growing teeth of pregnant rats rendered experimentally febrile, and in their young. The authors hesitate to describe the mechanism of injury. They point out that disturbances during the prenatal period may have a far greater effect on the fetal teeth than has been assumed. It is difficult to accept the changes in amelogenesis and dentinogenesis other than as the result of profound effects on the maternal metabolism, naturally and necessarily reflected in the fetus.

Johansen (p. 578) describes and illustrates a system for recording dental lesions in the living hamster. Universal use of one method of examination and recording should facilitate comparisons of results.

Hess, Lee, and Neidig (p. 585) report the amino acid composition of enamel protein. Having determined that enamel protein is an eukeratin, they demonstrated the presence of all twenty known amino acids except hydroxyproline* and hydroxylysine. Their analysis accounts for 98.3 per cent of the total nitrogen. This completes a long investigation and affords basic information for dental research workers in related fields.

H. B. G. R.

*Hydroxyproline has been identified in enamel by Stack.

THE EFFECT OF PENICILLIN DENTIFRICE ON THE CONTROL OF DENTAL CARIES

THOMAS J. HILL, D.D.S., JOHN SIMS, D.D.S., AND MILLICENT NEWMAN, B.S.

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OUR laboratory made a report[1] in 1949 on the use of a penicillin dentifrice in dental caries control in a Cleveland orphanage. The experiment was unregimented, using a powder containing 1000 units of penicillin per gram. The results of this one-year study did not show that this was an effective method of control of dental caries.

Since that time, two reports have appeared. Zander[2] in 1950, after a two-year study of a regimented program in a public school using a powder containing 500 units of penicillin per gram, showed a dental caries reduction of greater than 50 per cent. Walsh[3] of New Zealand in 1951 reported a two-and-one-half-year study of a small number of institutionalized children using 100 units per gram with negative results. Various other reports have appeared in the literature on the influence of the use of antibiotics on oral lactobacilli and other organisms in the mouth.

It was thought by us that perhaps the difference between the Zander experiment and the experiment reported from this laboratory was the difference between the regimented and unregimented control. Consequently, a second experiment was made. The present study was conducted in the Victory Park School at South Euclid, Ohio, and was patterned after the program used by Zander. The program was regimented as described in the discussion of method.

METHODS

The children brushed their teeth in school each day under the direct supervision of the teachers after there had been a program designed to train teachers in such supervision. From time to time, the brushing program was inspected by us. The children were also provided with toothbrushes and a dentifrice to be used at home. They were encouraged to brush their teeth at home twice daily.

There was a definite program of education provided for the children and parents. Talks were given to the P.T.A.'s on two occasions during the year; discussions also were held with the teachers to maintain their interest, and at several times motion pictures and other forms of oral health education were given to the pupils to stimulate cooperation.

This investigation was supported by a grant provided by the Bristol-Myers Company.
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The experiment started with 590 children and at the end of the experiment there were 492 who had been listed in the program throughout the year. The children were of ages 7 to 12 and came from the upper middle-class economic bracket.

The dentifrice used was a paste containing the procaine salt of penicillin. The freshly prepared paste dentifrice contained 1250 units per gram. There was some penicillin lost, but at no time did the penicillin level fall below 850 units per gram, and it perhaps averaged around 1000 units per gram.

All children in the school were given a number. Those numbers which ended in 3, 6, and 9 were used as controls, and all other members were used as experimentals. When siblings appeared in the school, they were numbered in such a manner that control and experimental children did not come from the same home. The division resulted in 347 in the experimental group and 145 in the control group.

At the beginning and at the end of the experiment dental examinations were made by one of us (J. S.) by the use of mouth mirror and exploring tine. Posterior bite-wing x-rays were used, and the records were prepared on charts designed for this purpose. Two salivary lactobacillus counts were made at the beginning and again at the end of the experiment.

The results are based upon the change that occurred during the one-year study. The results shown apply to DMF teeth, DMF surfaces, and total number of current lesions. In the computations, teeth which were lost during the year of study were considered to have had the same number of surfaces involved as was observed in the first examination.

FINDINGS

Table I shows age distribution, permanent teeth, noncarious permanent teeth, total number of cavities, total number of fillings, and the total number of surfaces involved at the beginning and at the end of the experiment.

From Table I, the results have been summarized in Table II. Some statistical difference results, depending upon the criteria used to measure the incidence of dental caries during the year of study. When DMF teeth are measured, the difference between the experimental and control groups is 9.74 per cent. When DMF surfaces are considered, the difference is 15.93 per cent, and when the total number of lesions occurring in the teeth during the year is measured, the difference is 14.52 per cent.

Regardless of the method of measurement, the experimental group had less tooth decay than the control group. However, when these figures are analyzed statistically, the only difference that is significant is the difference in decayed-missing-filled surfaces.

In Table III, the distribution of oral lactobacilli is shown at the beginning and at the end of the experiment. The counts were grouped into arbitrary classifications, and the percentages that fall into those classifications are shown. The difference between the experimental and control groups is not significant either at the beginning or at the end of the experiment.

TABLE 1

	EXPERIMENTAL									CONTROL								
AGE	NO.	PT	NCPT	TNC	TNF	MT	DFT	DFS 1950, DMFS 1951		NO.	PT	NCPT	TNC	TNF	MT	DFT	DFS 1950, DMFS 1951	YEAR
6	55	264	189	96	4	0	75	92		26	106	78	29	2	0	28	33	1950
		492	352	189	38	0	140	199			227	162	81	4	1	64	85	1951
7	71	634	445	259	48	0	189	272		29	267	181	119	32	0	86	138	1950
		836	595	315	139	1	240	393			353	236	196	67	0	117	248	1951
8	88	1059	761	412	162	0	298	539		33	388	280	150	67	0	108	194	1950
		1260	896	477	293	5	359	736			456	328	178	112	0	128	262	1951
9	53	732	533	292	124	0	199	373		25	339	228	137	80	0	111	196	1950
		953	707	319	208	1	245	502			446	316	171	111	1	129	272	1951
10	37	691	507	239	175	0	184	389		14	241	182	68	66	0	59	119	1950
		845	570	308	239	3	272	546			292	202	98	86	0	90	171	1951
11	34	778	526	315	153	0	252	420		13	295	192	116	79	0	103	175	1950
		870	553	354	224	4	313	564			331	201	149	89	0	130	225	1521
12	9	228	145	96	63	0	83	144		5	103	69	56	16	0	34	60	1950
		248	132	114	98	1	115	214			126	76	85	18	0	50	95	1951
Total	347	4386	3106	1709	729	0	1280	2229		145	1739	1210	675	342	0	529	915	1950
Total	347	5504	3805	2076	1239	15	1684	3154		145	2231	1521	958	487	2	708	1358	1951
						1699 DMFT									710 DMFT			

PT, Permanent teeth
NCPT, noncarious permanent teeth
TNC, total number caries
TNF, total number fillings
MT, missing teeth
DFT, decayed filled teeth
DFS, decayed filled surfaces
DMFS, decayed missing filled surfaces

TABLE II

DECAYED-MISSING-FILLED TEETH

	NO.	NCPT*	TEETH ERUPTED	TOTAL TEETH	TEETH THAT BECAME CARIOUS	PER CENT OF TEETH THAT BECAME CARIOUS	PER CENT OF DIFFERENCE
Experimental	347	3106	1118	4224	419	9.92	9.74
Control	145	1210	492	1702	187	10.99	

DECAYED-MISSING-FILLED SURFACES

	NO.	NCPTS†	SURFACES ERUPTED	TOTAL SURFACES	SURFACES THAT BECAME CARIOUS	PER CENT OF SURFACES THAT BECAME CARIOUS	PER CENT OF DIFFERENCE
Experimental	347	15530	5590	21120	925	4.38	15.93
Control	145	6050	2460	8510	443	5.21	

TOTAL LESIONS (CAVITIES AND FILLINGS)

	NO.	LESIONS 1950	LESIONS 1951	INCREASE IN LESIONS	AVERAGE INCREASE IN LESIONS PER PERSON	PER CENT OF DIFFERENCE
Experimental	347	2438	3315	877	2.53	14.52
Control	145	1017	1445	428	2.96	

*Noncarious permanent teeth.
†Noncarious permanent tooth surfaces.

TABLE III

LACTOBACILLUS COUNTS

NUMBER OF LACTOBACILLI		1950 (PER CENT)	1951 (PER CENT)
0 to 1,000	Experimental	26.7	29.2
	Control	26.6	22.8
1,000 to 10,000	Experimental	15.6	19.1
	Control	16.7	26.0
10,000 to 30,000	Experimental	10.9	11.1
	Control	7.7	12.3
30,000 to 50,000	Experimental	9.5	8.7
	Control	11.6	5.7
50,000 and over	Experimental	37.1	31.6
	Control	37.3	32.3

DISCUSSION

Several questions arise which are of interest. Inasmuch as this experiment was patterned after the Walpole, Mass., experiment, one wonders why the results were so different. It is understood that no two experiments in different localities and with different groups of people are identical, and minor differences must occur. In this experiment, the procaine salt of penicillin was used and in the Zander experiment the calcium salt was used. The procaine salt has a lesser solubility and also is a larger molecule. It would not seem probable that the relatively lesser solubility of the procaine salt would adequately explain the difference.

It is possible that the larger molecule of the procaine salt might have a lesser penetrability of the dental plaque. This has not been investigated. In this experiment, the children were a random selection and both the experimental and the control groups for each year or class were under the same teacher supervision. In the Walpole experiment the control and experimental groups were in separate schools.

The average increase in DMF surfaces in both the experimental and control groups is a constantly increasing figure for different ages. A variation in age distribution might be of some influence.

There were no clinical cases of allergic manifestations that developed during the experiment, although patch tests which were made on all the children at the end of the experiment indicated a minimal reaction in one individual. It is recognized that patch tests do not have a high degree of accuracy, but would be indicative if reactions were common. Tests were made to determine the possible development of resistant organisms, and some increases were observed.

CONCLUSIONS

1. The use of a paste dentifrice containing approximately 1000 units of the procaine salt of penicillin for one year on a regimented program gave a slight, but not a marked, reduction in the amount of dental caries.

2. These data indicate that a slight reduction in dental caries might be obtained from the regular and constant use of a dentifrice containing penicillin. However, the effectiveness is not of such a magnitude that it would be reasonable to expect that such a dentifrice, used irregularly by the public in an unregimented program, would be of material value in the reduction of the incidence of dental caries.

3. No significant change occurred in the salivary lactobacillus counts of the saliva.

4. Allergies did not present a clinical problem.

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THE DEVELOPMENT OF ORGANISMS WITH PENICILLIN RESISTANCE ASSOCIATED WITH THE USE OF A PENICILLIN DENTIFRICE

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THIS laboratory has reported on a study of the use of penicillin dentifrice in the control of dental caries in the children of the public schools of South Euclid, Ohio.[1] The program was regimented in so far as each child brushed his teeth with this penicillin dentifrice at school once every day and was urged to brush his teeth at home twice a day.

After one year's use of the dentifrice, the following study was made to determine if there had been an increase in the prevalence of organisms resistant to this antibiotic. There were 492 children in the experiment and the sensitivity study is based upon the results of tests on 814 organisms isolated from the saliva of 150 children chosen at random.

Two previous studies have been made on the development of resistant organisms resulting from the continuous use of a penicillin dentifrice. Following the Walpole, Mass., experiment, Lind and Zander[2] reported penicillin sensitivity tests on 259 organisms cultured from 293 children. Of these, 144 cultures were from the group using penicillin dentifrice, and 115 cultures were from the control group. From this study they concluded that the results from each group were sufficiently comparable to indicate that the constant use of penicillin dentifrice did not induce an acquired resistance in the streptococci and staphylococci isolated from these children. Later, Welch[3] conducted a further study on the Walpole experiment and his results indicated that there was a higher percentage of streptococci resistant to 0.5 unit or more of penicillin per milliliter in the mouths of the users of penicillin dentifrice than in the control group. Welch called attention to the fact that resistant organisms were found in the mouths of both groups.

The dentifrice used in this study was a paste containing the procaine salt of penicillin. The freshly prepared paste contained 1250 units per gram. There was some loss of penicillin, but at no time did its penicillin content fall below 850 units per gram. It is probable that the average content was close to 1000 units.

METHODS

The methods used in this experiment were as much like those of Welch as was practical from the material available. Cultures were made from the salivas of 150 children, 50 from the control group and 100 from the penicillin dentifrice users. Cultures were made originally from a larger number of individuals, but were lost during transfer, as *Streptococcus viridans* is some-

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times difficult to grow. Each salivary specimen was diluted 1-10 in sterile saline solution and streaked on a blood agar plate. After incubation, ten colonies were picked from each plate. Eight of the colonies were streptococci producing green hemolysis, and two were colonies which did not produce such hemolysis. Gram stains made on the isolated cultures showed those colonies other than streptococci included gram-positive and gram-negative cocci, and gram-positive and gram-negative rods. However, the largest number were gram-positive cocci. The organisms were transferred from the blood agar plate to tryptose agar slants, incubated, and then stored in a refrigerator. As fast as they could be handled they were transferred to tryptose broth with 2 per cent blood cells. After incubation each of these cultures was inoculated into six tubes of tryptose broth with 2 per cent blood cells. These tubes of broth contained, respectively, 0.0, 0.005, 0.02, 0.1, 0.5, and 1.0 unit procaine penicillin per milliliter. If the organisms grew in 1.0 unit of penicillin per milliliter, they were inoculated into tubes containing 5 and 100 units of procaine penicillin per milliliter. Growth was determined by the hemolysis of red cells.

In all, there were 814 cultures which survived the necessary transfers and are included in this report. Four hundred eleven of these cultures were strains of *Str. viridans* from the experimental group, 207 were strains of *Str. viridans* from the control group, 134 were organisms other than streptococci from the experimental group, and 62 were organisms other than streptococci from the control group. All cultures were checked for their Gram reaction and morphologic characteristics. When necessary the staining reaction and the morphology of organisms used in the penicillin tests were checked against the characteristics of the same organism in the original tryptose agar slants.

FINDINGS

Table I shows the results of the sensitivity tests of the *Str. viridans* to the varying concentrations of penicillin. This table shows the percentage of the total number of organisms which are susceptible to the amounts of penicillin per milliliter indicated, or the minimum concentration necessary to inhibit.

In a comparison of the users and nonusers of penicillin dentifrice the following facts are observed. Of the organisms that are more susceptible, the

TABLE I
THE PERCENTAGE OF STREPTOCOCCUS VIRIDANS INHIBITED BY THE CONCENTRATION OF PENICILLIN INDICATED

PENICILLIN USERS (411 ORGANISMS)		NONUSERS (207 ORGANISMS)	
UNITS OF PENICILLIN	PER CENT	UNITS OF PENICILLIN	PER CENT
.005	2.9	.005	1.0
.02	14.8	.02	21.7
.1	32.9	.1	41.1
.5	23.4	.5	18.4
1.0	9.7	1.0	4.8
5.0	11.7	5.0	8.7
100.0	4.6	100.0	4.3

nonusers, or the control group, have a higher percentage of organisms that are inhibited by 0.005, 0.02, and 0.1 unit of penicillin per milliliter. Of the more resistant organisms, the users of the penicillin dentifrice have a higher percentage of organisms which require 0.5 unit or more of penicillin in order to inhibit.

This indicates that a certain amount of resistance is developed by the *Str. viridans*, but the percentage of organisms which become resistant is not great. Of the organisms which require a concentration of penicillin of 0.5 unit or more to inhibit, there are 13.2 per cent more in the group using penicillin dentifrice than in the group who did not use penicillin dentifrice. It is also observed that the higher the concentrations of penicillin used, the less the difference between the users and nonusers of the dentifrice. The clinical significance of these differences is not clear.

TABLE II
THE PERCENTAGE OF ORGANISMS OTHER THAN STREPTOCOCCUS VIRIDANS INHIBITED BY THE CONCENTRATION OF PENICILLIN INDICATED

PENICILLIN USERS (134 ORGANISMS)		NONUSERS (62 ORGANISMS)	
UNITS OF PENICILLIN	PER CENT	UNITS OF PENICILLIN	PER CENT
.005	1.5	.005	4.8
.02	6.7	.02	8.1
.1	21.7	.1	16.1
.5	22.4	.5	37.1
1.0	20.9	1.0	17.7
5.0	16.4	5.0	9.7
100.0	10.4	100.0	6.5

In Table II similar figures are shown for the organisms not belonging to the *Str. viridans* group. These organisms were predominately gram-positive cocci and were slightly more resistant than the *Str. viridans*. A similar condition prevailed in this group where the penicillin users had a higher percentage of organisms requiring greater minimum concentrations necessary to inhibit. The significant difference is that the turning point between the users and nonusers came at the concentration of 1.0 unit per milliliter, whereas in the *Str. viridans* group it came at 0.5 unit per milliliter. The tables are similar in every other respect.

The data from Table I and Table II show graphically in Fig. 1 the trend in the minimum concentration necessary to inhibit both streptococcus and the

TABLE III
THE PERCENTAGE OF ALL ORGANISMS INHIBITED BY THE CONCENTRATION OF PENICILLIN INDICATED

PENICILLIN USERS (545 ORGANISMS)		NONUSERS (269 ORGANISMS)	
UNITS OF PENICILLIN	PER CENT	UNITS OF PENICILLIN	PER CENT
.005	2.6	.005	1.9
.02	12.8	.02	18.6
.1	30.1	.1	35.3
.5	23.1	.5	22.7
1.0	12.5	1.0	7.8
5.0	12.8	5.0	8.9
100.0	6.1	100.0	4.8

other organisms. Table III shows the minimum concentration necessary to inhibit all organisms (streptococci and other organisms) recovered from the users and nonusers of the dentifrice.

The actual percentage of organisms which were tested shows that a slightly larger percentage of resistant streptococci were in the mouths of the users of the penicillin dentifrice. Inasmuch as all of the organisms taken from

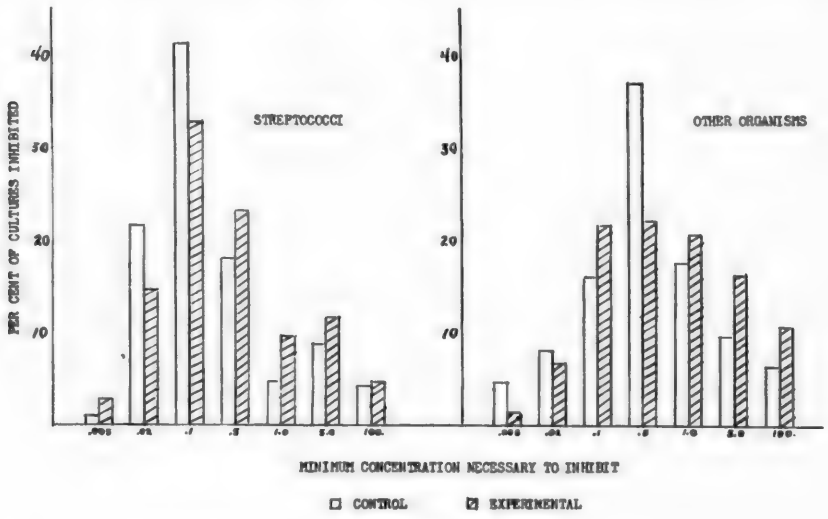

Fig. 1.

the blood agar plate did not survive transfers, this figure does not represent the percentage of individuals who have organisms resistant to higher concentrations of penicillin. Additional light may be shed upon this subject if we consider the percentage of individuals who have organisms resistant to 0.5 units or more of penicillin, rather than the percentage of organisms themselves. On this basis it was observed that 75 per cent of the users of penicillin had some *Str. viridans* resistant to 0.5 unit or more of penicillin, while in the control or nonusers of penicillin only 61.2 per cent had *Str. viridans* resistant to similar concentrations.

For the organisms other than the *Str. viridans,* 77.5 per cent of the penicillin users had organisms requiring minimum concentrations of 0.5 unit or more of penicillin per milliliter, and 75.3 per cent of the control group had similar organisms requiring similar amounts of penicillin to inhibit. The penicillin level above which more of these organisms were resistant occurred in the penicillin users at 1.0 unit per milliliter. We should more properly consider this as the turning point. Under these conditions there are 60.2 per cent of the users of penicillin who have organisms other than streptococcus-resistant, and there are 38.2 per cent of nonusers having similar resistant organisms. Although the numbers represented in these figures are smaller, more im-

portance might be attached to the fact that the difference between experimental and control groups was greater in the percentage of individuals than in the percentage of organisms involved.

CONCLUSIONS

1. The constant use of penicillin dentifrice increases the percentage of *Str. viridans* which require 0.5 unit or more of penicillin to inhibit.

2. There are similar increases in the percentage of organisms other than *Str. viridans* which required concentrations of 1.0 unit or more of penicillin per milliliter, to inhibit.

3. In the users of the penicillin dentifrice there was a greater percentage of individuals having resistant *Str. viridans* and other organisms than in the nonusers of the dentifrice.

4. These findings indicate that the production of resistant organisms by the constant use of a penicillin dentifrice may be of clinical significance.

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THE EFFECT OF SODIUM FLUORIDE APPLICATION ON THE DENTAL CARIES EXPERIENCE IN ADULTS

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EXTENSIVE clinical observations have been reported which indicate that sodium fluoride solutions applied topically will reduce the dental caries experience in children under the age of 16.[1-5] At the time our investigation was started only one study[6] on the topical application of sodium fluoride to the teeth of adults had been reported. This study indicated that there was no benefit derived by ninety-four young adults from the single application of an acidulated 0.5 per cent solution of sodium fluoride, using other individuals with a similar dental status as controls. Later, Klinkenberg and Bibby[7] reported that they were able to obtain a reduction of the caries experience in young adults by the topical application of sodium fluoride as great as, if not greater than, that obtained with children. Rickles and Beeks[8] reported similar percentages of dental caries reduction, treating fifty quadrants in twenty-five young adults with a neutral 2 per cent sodium fluoride solution.

MATERIALS AND METHODS

It was decided to use the method advocated by Knutson and Armstrong for applying the sodium fluoride solution. Primarily, it was believed that this method had been used the most to establish the benefit of topical fluoride applications for children. It was believed also that the only variable in our investigation should be the fact that the sodium fluoride solution was applied to the teeth of young adults instead of children. Another reason for choosing this method of application was that the treatment could be completed in about two weeks. This procedure, then, would make the method desirable for use by the armed services in basic training centers if the results of the study were found to be beneficial.

Beginning in March, 1948, and ending in June, 1949, 162 students at the University of Nebraska College of Medicine received sodium fluoride treatments and dental examinations. The 147 students who completed the experiment ranged from 20 years to 42 years of age. The mean age was 24.6 years and the median age was 24 years.

The student's treatment schedule was determined alphabetically with variations adjustable to class schedules. Each student was numbered in the order he presented himself. The students with odd numbers had the teeth in the upper and lower right quadrants treated with a 2 per cent solution of sodium

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fluoride, while those students with even numbers were treated on the upper and lower left quadrants. The nontreated areas served as control quadrants. A total of 324 quadrants were treated. The students were told that no other forms of fluorine medication should be used during the experimental period, but that any other form of caries control measures was permissible.

Treatments were scheduled at least two days apart. The treatments usually were completed in ten days, and at the most in fourteen days.

The examination (roentgenographic and visual), prophylaxis, and the sodium fluoride applications were conducted as follows: 1. At the first appointment the teeth were cleaned and the first sodium fluoride treatment was given. 2. At the second appointment two bite-wing roentgenograms were made, and the second sodium fluoride treatment was given. 3. At the third appointment the teeth were examined and charted, and the third sodium fluoride treatment was given. 4. At the fourth appointment the final sodium fluoride treatment was given.

The prophylaxis consisted of a thorough scaling of each tooth followed by the cleansing of all surfaces of the teeth with a prophylaxis paste. Rubber cups, brushes, and dental floss were employed for the cleaning process. The prophylaxis paste contained pumice, flour, precipitated calcium carbonate, starch, glycerin, and aromatic and sweetening agents. The mouth then was rinsed thoroughly.

The sodium fluoride was applied after isolating the entire mouth with ten 1½ inch No. 2 cotton rolls. Three cotton rolls were placed in the mucobuccal fold of the upper mouth, and three cotton rolls were placed in the buccal and labial folds of the lower mouth. The remaining four cotton rolls were placed in the floor of the mouth, two on each side of the tongue. The two cotton rolls on the control side of the tongue were held in place with a cotton roll holder. The teeth to be treated then were dried with compressed air. The mesial surfaces of the four first incisors were not treated. The sodium fluoride solution used was a neutral 2 per cent solution, although, through reaction with the glass container, the solution reached a pH of 8.1 after a year.

The sodium fluoride was applied to the teeth with No. 3 cotton pellets held in cotton pliers. The pellets were dipped in a dappen dish filled with about 2 c.c. of solution. The solution was applied first to the teeth in the lower arch and then to the teeth in the upper arch. A small explorer was used to force the solution into the fissures and sulci of the teeth. Dental floss then was used between the teeth to carry the solution into the interproximal surfaces. Care was taken that the floss and the contact areas were wet with solution to insure as much wetting of the interproximal surfaces as possible. For at least the next four minutes the mouth was held open and isolated while intermittent swabbing kept the treated side wet with solution. During these four minutes the cotton rolls that had become saturated (in only a few instances) were exchanged, one at a time, for dry cotton rolls. It was found, incidentally, that these cotton rolls could absorb at least 2 c.c. of any solution before becoming saturated. The

usual amount of solution used was 2 c.c. At the end of four minutes the cotton rolls were removed, and the mouth immediately was rinsed twice with water. A water syringe was used to facilitate the rinsing procedure.

The examination was made with a mouth mirror and a double ended No. 2 explorer under a dental light with the aid of compressed air. Bite-wing roentgenograms of the posterior teeth were available for referral at the time of examination. Transillumination was used in examining the anterior teeth.

A complete charting of the teeth was accomplished at the third appointment. All restorations on each tooth surface were recorded in such a manner as to indicate their size, location, and type of material used. All extracted teeth and all missing or unerupted teeth were charted.

All carious areas of enamel on each surface were charted separately and graded, according to the degree of caries activity, from 5 to 1. A grading of 5 denoted a carious area where the caries had just extended into the dentin, while a grade of 1 meant a carious area approximating the pulp. All other designations, with or without plus or minus suffixes, denoted intermediate stages of caries activity. A definite "catch" by a sharp explorer often was used as a criterion in doubtful cases. Those doubtful areas not having a definite "catch" to the explorer but presenting enamel faults were labeled by either the word "etching" or a "5+." Pulpal exposures were designated by "1–." A grading of 3 indicated the intermediary or average stage, i.e., about halfway between pulp and the dentinoenamel junction. At the time of re-examination the chart was checked for new restorations and, if present, they were charted as before. All old and new carious areas were designated as previously charted to denote the extent of caries activity. All teeth that were extracted or had erupted since the first examination also were charted. Mesial surfaces of the central incisors were not tabulated because of the inability to isolate them during treatment.

Re-examination of the 147 students who completed the course of treatments was started in the spring of 1949 and completed in June 1950. The mean time elapsed between the first treatment and the second examination was 13.6 months, with the median time being 13 months. The examiner had no knowledge as to which side was treated and the element of unintentional knowledge was eliminated by the fact that the number treated on the right quadrants was equal to the number treated on the left quadrants. The same examiner did both the first and second examinations.

RESULTS

The effects of the topical application of a 2 per cent sodium fluoride solution on dental caries incidence in 147 young adults after 13 months had elapsed are shown in Tables I and II. Table I shows that there were sixty-five new decayed or filled teeth in the 294 treated quadrants and sixty-two new DF teeth in the 294 nontreated quadrants. This is a total of 127 new DF teeth in 147 individuals. There were 194 new DF surfaces in 147 individuals, of which 103 surfaces were in 294 treated quadrants and 91 surfaces were in 294 nontreated quadrants. Table I indicates that the applications were of no significant value in decreasing dental caries incidence after 13 months.

TABLE I
EFFECT OF 2% SODIUM FLUORIDE SOLUTION ON TOTAL NEW DF TEETH AND NEW DF SURFACES

147 INDIVIDUALS	NUMBERS OF NONCARIOUS TEETH FIRST EXAMINATION	NEW DF TEETH SECOND EXAM.	DF SURFACES IN NEW DF TEETH	NEW DF SURFACES IN PREVIOUSLY CARIOUS TEETH	TOTAL NEW DF SURFACES
294 treated quadrants	1698	65	72	31	103
294 untreated quadrants	1664	62	68	23	91

Table II presents the effect of sodium fluoride applications in terms of the number of individuals that may or may not have benefited from the applications. Forty-six individuals had no new carious areas in any quadrant (or forty-seven individuals had no new carious surfaces in any quadrant). It could not be assumed whether these individuals did or did not benefit from the sodium fluoride applications. Forty-eight individuals had less carious areas in the treated quadrants (or forty-seven individuals had less carious surfaces in the treated quadrants). This group of forty-seven (or forty-eight) appears to have derived benefit. On the other hand, fifty-three individuals had the same or more new carious areas in the treated quadrants or fifty-three individuals in terms of new carious surfaces. This group of fifty-three appear to have derived no benefit. On this basis, therefore, there is little, if any, value in reducing the incidence of dental caries in this age group by the topical application of a 2 per cent solution of sodium fluoride.

TABLE II
CLASSIFICATION OF 147 INDIVIDUALS ON THE BASIS OF SODIUM FLUORIDE EFFECT ON CARIES INCIDENCE

GROUPS	NEW CARIOUS SURFACES (INDIVIDUALS)	NEW CARIOUS AREAS (INDIVIDUALS)
Group I	47	46
Group II	17	14
Group III	36	39
Group IV	47	48
Total	147	147

I. Those individuals having no new caries on any quadrant.
II. Those individuals having the same amount of caries on both quadrants.
III. Those individuals having more caries in the treated quadrants.
IV. Those individuals having less caries in the treated areas.

DISCUSSION

In evaluating this report, it should be noted that the methods used were essentially those used by Knutson and Armstrong.[2-4] Alcohol was not used to dry the teeth as was done by Klinkenberg and Bibby,[7] and no wetting agent was used as was used by Rickles and Becks.[8]

Although the same examiner made both the initial and second clinical and roentgenographic examinations, it is recognized that different interpretations of caries incidence might be made in questionable instances by even the same examiner. The fact that the examiner had no clues whatsoever as to which side

was treated and that the degree of caries or any enamel etchings were recorded at the initial examination contributed toward a more objective evaluation of doubtful or questionable new areas of decay.

It is believed that caries control studies which use the mouth of the individual for both experimental and control fields and assume, therefore, that the caries control measure is the only variable in the experiment, would be evaluated more accurately if interpreted in terms of benefit to the individual. Such an evaluation also would be helpful to the practitioner who wishes to inform his patients of the benefit of the measure. The lumping together of results in terms of total areas of caries in the total number of teeth also lumps together the many variables that might exist between individuals, and thereby presents a distorted picture of the value of the method.

SUMMARY

1. One hundred forty-seven medical students, 20 to 42 years of age (median age 24 years), had two quadrants in their mouths treated with a 2 per cent solution of sodium fluoride. Fifty per cent of the students received treatments on the right quadrants and the other fifty per cent were treated on the left quadrants. The opposite quadrants in each case served as controls. Clinical and roentgenographic examinations were made before the last sodium fluoride treatment was given and again approximately 13.5 months later. The same examiner made both examinations.

2. Evaluation of new caries in 294 treated quadrants and 294 nontreated quadrants showed no significant difference in the dental caries incidence.

3. On the basis of total new caries, or on the basis of each individual's results, there was no significant reduction in the dental caries incidence.

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A PROCEDURE FOR THE ROUTINE DETERMINATION OF FLUORINE IN POTABLE WATERS CONTAINING IRON, MANGANESE, ALUMINUM, AND CHLORINE

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BY THE first of the year 1952, 158 communities in thirty-three states were fluoridating their water supplies.[1] It has been estimated that a total of 34 million people will be drinking fluoridated water by the end of 1952.

Epidemiological studies have established that 1.0 to 1.2 parts per million of fluoride ion naturally occurring in communal water supplies reduces the incidence of tooth decay by 50 to 60 per cent without producing mottled enamel. Because mild dental fluorosis may occur at a slightly higher concentration of fluoride ion and maximum protection against caries may not be attained if the fluoride ion concentration is too low, it is of the utmost importance that the fluoride ion be supplied and maintained at the proper level in the communal water supply. Thus, the need for a simple, rapid, and accurate method for the routine determination of fluoride is apparent.

The purpose of this article is to report a method for the routine determination of fluorine in potable waters which may contain various substances that interfere in the conventional Sanchis-Scott method[2] of analysis. The procedure being presented, which is a modification of the Sanchis-Scott method, permits the determination of fluoride ion in potable waters containing iron, manganese, aluminum, and chloride, as well as the substances tolerated by the Sanchis-Scott method. The unmodified Sanchis-Scott method permits the presence of chloride, sulfate, alkalinity (expressed as $CaCO_3$), and phosphate, all in micro quantities. The employment of this modified Sanchis-Scott method of analysis for routine control of fluoride in communal water supplies will circumvent the use of the more laborious and time-consuming distillation method for the separation of the fluoride from interfering ions.

INTERFERENCE BY IRON

Many communal water supplies contain iron as a natural contaminant, while others contain this element as the result of water purification involving the addition of ferrous sulfate as a flocculating agent.

The results of an investigation of the interference of iron in the Sanchis-Scott fluoride method are shown in Table I.

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A portion of this material was taken from a thesis submitted to the Graduate School, Indiana University, for the partial fulfillment of the requirements for the degree, Master of Arts.
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TABLE I

INTERFERENCE BY FERRIC IRON IN THE SANCHIS-SCOTT METHOD OF FLUORIDE ANALYSIS (SPECTROPHOTOMETRIC)

Fe^{+3} PPM	PPM F^- RECOVERED WHEN 0.50 PPM F^- TAKEN	PPM F^- RECOVERED WHEN 1.00 PPM F^- TAKEN
0.1	0.50 ± 0.02	1.00 ± 0.02
0.5	0.48	0.96
0.9	0.44	0.92
1.5	0.41	0.86
2.0	0.36	0.83
2.5	0.26	0.77
3.0	0.19	0.72

Due to the high acidity of the reaction medium in the zirconium-alizarin method of fluoride analysis, the iron is in solution predominately as the ferric ion. Ferric iron exhibits a strong tendency to combine with fluoride ions to form a stable complex, FeF_6^{-3}, making some of the fluoride unavailable for bleaching of the zirconium-alizarin lake.

Since the bleaching of the zirconium-alizarin lake is considered to be based upon the reaction

$$\underset{\text{(Red)}}{\text{Zr – alizarin lake}} + \underset{\text{(colorless)}}{6\ F^-} \rightarrow \underset{\text{(colorless)}}{ZrF_6^{-2}} + \underset{\text{(yellow)}}{\text{alizarin}}$$

it is reasonable to assume that the ferric iron interferes with this bleaching of the lake by competing with zirconium for fluoride ions, thus giving low results for fluoride.

The tendency of an ion to form complexes is a function of its ionic potential (ratio of ionic charge to radius). By reducing the iron from Fe^{+3} to Fe^{+2}, the charge is decreased and the radius is increased. This means a reduction in ionic potential from 5.00 for ferric iron to 2.67 for ferrous iron with a corresponding decrease in tendency to complex with fluoride and interfere with the bleaching of the lake.

In the present investigation it was found that thioglycolic acid (mercaptoacetic), $HSCH_2COOH$, will readily reduce ferric iron to the ferrous state and at the same time complex the iron, possibly as the inner complex salt, ferrous thioglycollate, $Fe(HSCH_2COO)_2$.

When samples of waters containing both fluoride and ferric iron were treated with thioglycolic acid prior to analysis, the iron did not interfere up to 25 ppm of ferric ion, as shown in Table II.

TABLE II

RECOVERY OF FLUORIDE IN THE PRESENCE OF IRON USING THE THIOGLYCOLIC ACID MODIFICATION

Fe^{+3} PPM	PPM F^- RECOVERED WHEN 1.00 PPM F^- TAKEN
2	1.00
3	1.00
5	1.00
15	1.00
25	1.00

INTERFERENCE BY MANGANESE

Manganese present in potable waters probably exists as hydrous manganese dioxide. It has been reported[3] that manganese interferes with the zirconium-alizarin fluoride method by way of oxidation of the organic anion of the lake by the colloidal manganese dioxide, which is a powerful oxidizing agent. Oxidation of the dye results in bleaching of the lake and in high fluoride analysis.

Colloidal managnese dioxide solutions were prepared by reducing ammoniacal solutions of potassium permanganate with ethanol.

The influence of colloidal manganese dioxide upon the Sanchis-Scott method for fluoride analysis is shown in Table III.

TABLE III

INTERFERENCE BY HYDROUS MANGANESE DIOXIDE IN THE SANCHIS-SCOTT METHOD OF FLUORIDE ANALYSIS (SPECTROPHOTOMETRIC)

MnO_2 PPM	PPM F^- RECOVERED WHEN 0.00 PPM F^- TAKEN	PPM F^- RECOVERED WHEN 0.50 PPM F^- TAKEN	PPM F^- RECOVERED WHEN 1.00 PPM F^- TAKEN
0.05	0.00	0.50	1.00
0.10	0.00	0.50	1.00
0.20	0.20	0.69	1.11
0.30	0.41	0.84	1.20
0.40	0.57	1.00	1.32
0.50	0.84	1.13	1.44

Taras, Cisco, and Garnell[3] found that the interference by manganese on the zirconium-alizarin fluoride method could be eliminated by reducing the manganese dioxide to the manganous ion, for which purpose they employed hydrogen peroxide. Thioglycolic acid readily reduces tetravalent manganese to the manganous ion in the strongly acid medium, thus removing the interference by this element in the standard method as shown in Table IV.

TABLE IV

RECOVERY OF FLUORIDE IN THE PRESENCE OF MANGANESE DIOXIDE USING THE THIOGLYCOLIC ACID MODIFICATION

MnO_2 PPM	PPM F^- RECOVERED WHEN 1.00 PPM F^- TAKEN
1	1.00
2	1.00
3	1.00
5	1.00
15	1.00
25	1.00

INTERFERENCE BY ALUMINUM

The aluminum ion may be a constituent of municipal water supplies as a natural contaminant, or due to the use of aluminum sulfate in water purification. Not all of the aluminum is necessarily removed through coagulation of the hydrous oxide during the purification process.

The nature of the interference of aluminum ions, upon the zirconium-alizarin method is one of simple complex formation between the aluminum and fluoride ions, both of which have very high ionic potentials. The lowering of

the fluoride ion concentration of the sample by aluminum complexation decreases the bleaching of the lake by fluoride and leads to low results for the fluoride analysis.

The results of a spectrophotometric investigation of the interference of aluminum is shown in Table V.

TABLE V

INTERFERENCE BY ALUMINUM IN THE SANCHIS-SCOTT METHOD OF FLUORIDE ANALYSIS (SPECTROPHOTOMETRIC)

Al^{+3} PPM	PPM F^- FOUND WHEN 0.00 PPM F^- TAKEN	PPM F^- FOUND WHEN 0.50 PPM F^- TAKEN	PPM F^- FOUND WHEN 1.00 PPM F^- TAKEN
0.00	0.00	0.50	1.00
0.10	0.00	0.50	1.00
0.30	0.00	0.48	0.98
0.50	0.00	0.48	0.96
0.90	0.00	0.48	0.90
1.50	0.00	0.45	0.83
2.00	0.00	0.40	0.78
2.50	0.00	0.37	0.74
3.00	0.00	0.35	0.70

No effective method for eliminating the interference exhibited by aluminum is available except the distillation of the fluoride from the interfering aluminum ion.

Particular note should be made of the fact that the extent of interference shown by aluminum is a function of the aluminum concentration. This relationship may be used to advantage in determining the actual fluoride content of the water when aluminum is present. The method is explained under Analytic Procedure.

INTERFERENCE BY CHLORINE

The elementary chlorine[3] or its hydrolysis products in chlorinated municipal waters interferes with the zirconium-alizarin method by oxidizing the organic anion of the lake, thus bleaching it, and leading to high fluoride results. Thioglycolic acid readily reduces chlorine or hypochlorite to chloride, which does not interfere.

ANALYTIC PROCEDURE FOR THE DETERMINATION OF FLUORIDE ION IN THE PRESENCE OF IRON, MANGANESE, ALUMINUM, AND CHLORINE

REAGENTS

Zirconium-Alizarin Reagent.[4]—Dissolve 0.3 Gm. of zirconium oxychloride ($ZrOCl_2.8H_2O$) in 50 ml. of distilled water (all distilled water used should be fluoride-free) contained in a one liter flask. Dissolve 0.07 Gm. of sodium alizarin sulfonate in 50 ml. of distilled water and pour slowly into the zirconium oxychloride solution, while swirling the flask. Dilute 112 ml. of analytic reagent hydrochloric acid (sp. gr. 1.19) to 500 ml. with distilled water. Add 37 ml. of analytic reagent sulfuric acid (specific gravity 1.84) to 400 ml. of distilled water and dilute to 500 ml. with distilled water. After cooling, mix the two acid solutions. To the zirconium-alizarin solution in the one liter flask, add the mixed acid solution to the mark and mix. The reagent changes color from red to yellow in an hour and then is ready for use.

Standard Sodium Fluoride Solutions.—Dissolve 0.221 Gm. of spectrographically pure sodium fluoride in distilled water and make up to a liter. Dilution of 100 ml. of this stock solution to one liter gives a solution containing 0.01 mg. of fluoride ion per milliliter.

Thioglycolic Acid.—74.6 per cent acid from Evans Chemetic, Inc.

PROCEDURE

Standards for visual comparison are 0.00, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.08, 0.10, 0.12, and 0.14 mg. of fluoride made up to 100 ml. with fluoride-free distilled water, and contained in 100 ml. Nessler tubes.

Each sample of water to be analyzed is determined in duplicate as directed below unless aluminum is known to be absent, in which case single samples are adequate.

If the presence or absence of aluminum is unknown, a 100 ml. aliquot of the water sample is pipetted into a 250 ml. beaker, and a 50 ml. aliquot of the same sample is placed in another beaker. This is done for each water sample to be analyzed.

The beakers containing the samples are heated on a hot plate until a temperature of 70° C. to 80° C. is reached, and then three drops of thioglycolic acid are added to each sample and the beakers are removed from the hot plate immediately. The samples are poured into 100 ml. Nessler tubes and allowed to cool to room temperature. The 50 ml. samples are diluted to 100 ml. with distilled water, and 5 ml. of the zirconium-alizarin reagent is pipetted into each of the Nessler tubes. Five milliliters of the zirconium-alizarin reagent is also added to each of the standard fluoride solutions covering the desired concentration range. Mix the samples and compare visually after one hour.

In the case of spectrophotometric comparisons, 10.0 cm. cells and a wave length of 5250 Å are used. The greatest change in transmittancy occurs within the first hour, so the spectrophotometric measurements are made after the samples have reacted for one hour. Comparison of a sample to a standard is only reasonably valid if the two measurements are made within ten minutes of each other.

Null the 0.14 mg. fluoride sample at 100 per cent transmission and determine the per cent transmission of the remaining standards and samples.

The fact that the degree of interference shown by aluminum varies regularly with the aluminum ion concentration as shown in Table V makes it possible to determine fluoride in the presence of aluminum. For example, suppose a sample of water actually contains 1.00 ppm of fluoride and 2.00 ppm of aluminum. A 100 ml. aliquot would analyze as containing 0.78 ppm fluoride (see Table V), while a 50 ml. aliquot of the same sample diluted to 100 ml. will analyze as containing 0.48 ppm of fluoride. Since the fluoride concentration is halved by taking a 50 ml. aliquot, the fluoride recovered should be just one-half that determined using the 100 ml. aliquot, or 0.39 ppm of fluoride. Due to the lower interference of aluminum at its lower concentration in the 50 ml. aliquot, 0.48 ppm of fluoride is recovered. Converted to double the concentration, as would be the case for a 100 ml. aliquot, the corrected analyses for fluoride would be 0.96 as compared to an actual concentration of 1.00 ppm.

In case the water to be analyzed contains more than 1.5 ppm fluoride, an aliquot smaller than 100 ml. may be taken for analysis to bring the fluoride concentration within the determinable range. If the concentration of fluoride in the water sample is less than 0.3 ppm, concentration by evaporation may be used to bring the fluoride concentration into the 0.3 to 1.5 ppm range covered by the Sanchis-Scott method.

Tap water samples from Indianapolis and Bloomington, Indiana, were analyzed for fluorine by the modified Sanchis-Scott method described above. The results of these analyses checked closely with those obtained by the Willard and Winter distillation procedure for the removal of interfering ions.

SUMMARY

The Sanchis-Scott method for the determination of micro amounts of fluoride in potable waters has been modified to permit accurate analysis for fluoride in the presence of iron, manganese, aluminum, and chlorine, which substances interfere in the unmodified Sanchis-Scott method. Chloride, sulfate, alkalinity (expressed as carbonate), and phosphate are tolerated to the same limiting concentrations for noninterference as in the original Sanchis-Scott method.

The modification involves a preliminary treatment of the water sample being analyzed for fluoride by the conventional Sanchis-Scott method with thioglycolic acid. Ferric iron, manganic manganese, and elementary chlorine are reduced to ferrous iron, manganous manganese, and chloride ion, respectively. In the reduced condition, these elements do not interfere with the accurate determination of the fluoride ion by the conventional method.

The fact that the interference of aluminum in the Sanchis-Scott method of fluoride analysis decreases with decreasing concentration of the aluminum ion makes it possible to determine the fluoride accurately by the proper dilution of the sample taken for analysis.

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EFFECT OF CERTAIN CONCENTRATIONS OF FLUORIDE ON ENAMEL AND DENTIN AS FORMED IN TRANSPLANTS OF TOOTH GERMS AND RELATED STUDIES

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THE mechanism of fluorides in caries inhibition has not yet been completely established. In the Evanston Caries Study it was shown by Hill, Blayney, and Wolf[12] that the rate of caries was lowered in 6-, 7-, and 8-year-old children. After receiving fluorides, the greatest effect seemed to be on the permanent teeth and not on the deciduous teeth. Evidence has not been conclusive, however, that fluorides reduce the permeability of the teeth[22] and the solubility of enamel,[16, 19, 29] or lower salivary lactobacillus counts.[2, 13, 26] In a study on the effect of fluoride on calcium and phosphorus metabolism in rats, Lantz and Smith[14] found that there was retardation both of growth and of calcification in young animals. Recent work of Paff and Boyd[24] with developing bone in tissue cultures in the presence of fluorides supports the previous investigators' observations. Goldenberg and Sobel[8] found that both fluoride and cyanide ions are effective inhibitors of calcification in vivo, but not in vitro, when additional magnesium is available. It has been suggested recently by Robinson[25] and Neuman, Neuman, Main, O'Leary, and Smith,[21] in studies on the surface chemistry of bone, that the fluoride ion competes with other compatible ions for a place on the periphery of the hydroxy-apatite molecule. The work of Gerould[6] on the electron microscopic study of enamel rods inadvertently showed, among other things, that the cuticular structure surrounding these rods was changed in fluoride-treated animals. Significantly, McClure reported that the hair, nails, and other protective organs are rich in fluorides.[17] Stein, Eggleston, Hems, and Krebbs[28] observed that NaF acts as an inhibitor by interfering with the production of energy-producing systems. Likewise, Lehninger[15] and Najjar[20] found that fluoride complexes with Mg ++ and causes inhibition of enolase in the presence of inorganic phosphate.

It is the purpose of this report to show the effects of certain concentrations of fluorides on dentin and enamel as formed in transplants of tooth germs. To better illustrate the mechanism of fluorides, part of this presentation includes a comparative study of the action of fluorides on transplanted tooth germs and embryonic lung tissue that have been impregnated with methylcholanthrene. In addition, there is included also an account of the effect of fluorides on the ameloblasts of the incisor teeth of Norwegian white rats. With this comprehensive approach, it is felt that a more definite understanding of its mode of action may be given.

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MATERIALS AND METHODS

In the present experiments fluorides were administered either by drinking water or by intermittent interperitoneal or intramuscular injections. The animals used were open-stock guinea pigs, Norwegian white rats and dba mice. All were healthy young adults, about evenly divided as to sex. Tooth germs were obtained from embryos of a known age (from dba mice and from guinea pigs). Guinea pigs received either intraocular or intracerebral transfers of tooth germs. Mice usually received axillary transfers, with an occasional group receiving brain transfers. Rats did not receive any transfers, but the effects of fluoride on the ameloblasts of the incisor teeth were studied. Adequate controls were carried for each series of experiments. Guinea pigs received fluorides either by injection or by drinking water. Mice usually received fluorides by drinking water. Rats received calcium fluoride by injection. In cases where methylcholanthrene was used one group of animals received fluorides in their drinking water or by injection, while the other group was given untreated drinking water or did not receive injections.

Recovered sections were fixed in formalin, decalcified, and stained with hematoxylin and eosin before sectioning.

Fluoride solutions were made up as follows: 1 Gm. of CaF_2 or NaF was dissolved in 1,000 c.c. of distilled water. This comprised the stock solution and from it other dilutions were made. Twenty cubic centimeters of the stock solution diluted to 1,000 c.c. with distilled water gave a 1:50,000 solution, or 13.3 c.c. of the stock solution diluted to 1,000 c.c. gave a 1:75,000 solution. These solutions were used for drinking water. Since the solubility constant of CaF_2 is 0.0017 Gm. per 100 c.c. at 26 degrees C., it was not entirely soluble in the stock solution, but appeared more soluble in the dilutions used in drinking water. All solutions were stored at 10 degrees C. in polyethylene flasks when not in use. When injections were made, 3/20 c.c. of the stock solution was injected into the thigh muscles of guinea pigs and interperitoneally in rats. (1 c.c. of stock solution had 1,000 μg of either CaF_2 or NaF. 1 c.c. of 1:50,000 dilution had 20 μg of either CaF_2 or NaF. 1 c.c. of 1:75,000 dilution had 13.44 μg of either CaF_2 or NaF.)

Technics for obtaining and transplanting tooth germs to the various sites have been described previously.[3, 4]

The two divisions of these experiments refer to the animals used as hosts and are the basis for Tables I and II. Table I deals with mice and rats, Table II with guinea pigs.

Tooth germs from embryos of a known age were transplanted to the various locations as indicated in Tables I and II. Other tissue used was embryonic mouse lung which was transplanted to the axillae of mice. The comparative effect of methylcholanthrene on transplants of tooth germs and on embryonic lung transplants was studied with and without fluorides.

Ten dba mice used 16 ounces or 1,250 c.c. of water every seven days; about one-half was lost by evaporation and waste. It was estimated that each mouse consumed about 16 drops, or 1 c.c., daily. This was the equivalent of 20 or 13.44

TABLE I. MICE

NUMBER OF ANIMALS	SEX	FLUORIDE	TISSUE TRANSPLANTED	SITE	DURATION (DAYS)	OBSERVATIONS AND FINDINGS
23 dba	both	CaF_2 1:50,000 oral	dba tooth germs from 13 day embryos	axillae	34-50	Delay of calcification, enamel and dentin laid down but some cartilage associated with dentin
21 dba	both	NaF 1:50,000 oral	dba tooth germs from 13 day embryos	brains	20-55	Growth delayed no calcification of enamel and dentin by 55 days
20 dba	both	none controls	dba tooth germs	brains	20-55	Calcified enamel and dentin found by 25 days
20 dba	both	NaF 1:75,000 oral	dba tooth germs from 13 day embryos	axillae	60-100	By 100 days there was some calcification, but not much attempt at revision
25 dba	both	13 c* MC† and NaF 12 NaF 1:75,000 oral	Guinea pig tooth germs from 28 day embryos	axillae	60-100	Some cartilage in pulpal areas, no effect with MC on tooth sturcture, no keratin
60 dba	both	30 NaF 1:50,000 30 CaF_2 1:50,000 oral	dba tooth germs from 14 day embryos	axillae	3-75	Calcification of tooth structures retarded, not many cells in pulpal areas. Effect seemed the same with both solutions. No revision
60 dba	both	none controls	dba tooth germs from 13 and 14 day embryos	axillae	30-100	Calcified tooth structures found, revised tooth structure frequently found after 45 days
40 dba	both	20 c MC and NaF 20 1:50,000 oral	dba tooth germs from 14 day embryos	axillae	70	No keratin found with MC, calcification delayed in all, transplants without MC smaller and in females with MC transplants smallests
40 dba	both	20 c MC and NaF 1:50,000 oral 20 c MC	dba embryonic lung from 12 day embryos	axillae	72	Intra-alveolar proliferation in both but more pronounced in absence of NaF; with MC alone, filling of alveoli with squamous cells, epithelial pearls, and also keratin associated with transplants
12 white rats	both	3/20 c.c. 1:1,000 CaF_2 daily injections up to 28	none	none	10-60	Animals sacrificed intermittently and effect observed on ameloblasts, cell walls thickened, enamel and dentin altered, after 20 injections severe effects on ameloblasts but only slight loss of color observed even after 28 injections and an elapsed time of 60 days

Note: Transplanted tissue recovered in better than 90 per cent of axillary transfers; in brain or eye transfers, this averaged about 70 per cent. An increased number of lymphocytes was found near transplants in all fluoride-treated animals.

*c: with

†MC: methylcholanthrene

TABLE II. GUINEA PIGS

NUMBER OF ANIMALS	SEX	FLUORIDE	TISSUE TRANSPLANTED	SITE	DURATION (DAYS)	OBSERVATIONS AND FINDINGS
33	19 M 14 F	NaF 1:50,000 oral	G.P. tooth germs from 21 day embryos	Brains	10-90	Development of enamel and dentin without complete calcification even up to 90 days, no revision of dentin
28	14 M 14 F	CaF_2 1:50,000 oral	G.P. tooth germs from 21 day embryos	Brains	35	Calcification retarded, ameloblasts altered
20	10 M 10 F	NaF 1:75,000 oral	G.P. tooth germs from 23 day embryos	Eyes	28-55	Calcification of enamel and dentin delayed, cartilage associated with dentin
20	10 M 10 F	CaF_2 1:75,000 oral	G.P. tooth germs from 23 day embryos	Eyes	28-55	Calcification of enamel and dentin delayed, structure of enamel altered
20	10 M 10 F	none controls	G.P. tooth germs from 23 day embryos	Eyes	10-90	Calcified tooth structures formed, also revised dentin seen in transplants that remained in place for longer periods
20	10 M 10 F	CaF_2 1:50,000 oral 10 c* MC,† 10 s‡ MC	G.P. tooth germs from 25 day embryos	Brains	20-90	No keratin formation with MC, calcification delayed in both
20	10 M 10 F	NaF 1:50,000 oral 10 c MC, 10 s MC	G.P. tooth germs from 23 day embryos	Eyes	20-53	Dentin did not revise itself into osteoid or bonelike tissue, instead cartilage was often formed. No keratin with MC. Ameloblast cell walls altered
8	4 M 4 F	NaF oral 1:50,000	G.P. tooth germs from 25 day embryos	Eyes	20-55	Delay of calcification, ameloblasts had not keratinized by 55 days
8	4 M 4 F	CaF_2 oral 1:50,000	G.P. tooth germs from 25 day embryos	Eyes	20-54	As above not any apparent difference between NaF and CaF_2
16	8 M 8 F	none controls	G.P. tooth germs from 24 day embryos	Eyes	20-53	Calcified structures formed, with revision of dentin and obliteration of pulpal areas in most cases
14	7 M 7 F	CaF_2 3/20 c.c. daily 1:1,000 up to 15 injections	G.P. tooth germs from 22 day embryos	Eyes	20-40	Cell walls of ameloblasts primarily altered, delay of calcification, not as much effect on odontoblasts
16	8 M 8 F	NaF 3/20 c.c. daily 1:1,000 up to 25 injections	G.P. tooth germs from 22 day embryos	Eyes	20-45	Results much the same as above no apparent difference between NaF and CaF_2
8	4 M 4 F	NaF 3/20 c.c. daily 1:000 up to 17 injections	G.P. tooth germs from 23 day embryos	Brains	46	Delay of calcification, cartilage associated with dentin formation, ameloblasts altered
8	4 M 4 F	CaF_2 3/20 c.c. daily 1:000 up to 17 injections	G.P. tooth germs from 23 day embryos	Brains	46	As above

A greater number of lymphocytes was found in association with transplants when fluorides were used.

*s: without
†MC: methylcholanthrene
‡c: with

μg of fluorides daily, depending on the concentration used in drinking water. Ten guinea pigs weighing 400 to 600 Gm. easily used 1,250 c.c. daily, and here too about one-half was actually consumed. Thus, each pig received approximately 1,200 or 706.40 μg of fluorides daily, depending upon the strength of the solution. They tolerated this amount very well over long periods, with no gross alteration in the incisor teeth. Guinea pigs and rats, when injected, received 150 μg daily.

The weight of animals was checked biweekly, and it was found that when drinking water was given with the previously described dilutions of fluorides the weight of these animals compared favorably with the controls. Weight of animals receiving injected fluorides was likewise compared, and there were no significant changes. Transplants were given several days to establish themselves in the new environment before the administration of fluorides.

RESULTS

Development of tooth germs was retarded regardless of the transplantation site. The several concentrations of fluorides used did not appear to have different end results. Usually calcified enamel and dentin were found in tooth germ transplants after 21 to 28 days.[3] Where fluoride had been administered, even after 60 to 90 days, maturation of enamel and dentin was delayed. With this delay of calcification, the thickness of uncalcified enamel and dentin appeared increased, and these structures also appeared more homogeneous than the controls. The enamel layer stained a very dark purplish blue and the dentin stained a lighter purplish pink.

The incisor teeth of rats that received daily injections of 150 μg CaF_2 (3/20 c.c. of 1:1,000 solution of CaF_2) were studied. The striking observations of Schour and Smith[27] were lacking, but it must be remembered that they used a stronger solution (0.3 c.c. of 2.5 per cent NaF) than that employed in the present instance. Normal ameloblasts are seen in Fig. 1. After ten injections, which was the equivalent of 2,500 μg of fluoride, the cell walls of the ameloblasts became considerably thickened. Fig. 2 shows ameloblasts after ten injections. After thirteen injections nucleoli were still evident in the ameloblasts. They appeared to separate from each other and, except for a slight loss of polarity, there did not seem to be much change. The Golgi apparatus at this time was less definite than with twelve injections. Fig. 3 shows ameloblasts after fifteen injections. By twenty injections the ameloblasts had lost their polarity, the nucleoli were less definite, the nuclei appeared to elongate, there were a number of vacuoles, and the Golgi apparatus, in most instances, had disappeared. It was noted also that there were was a great increase of vascularity in the tooth follicle. There also was an increase, both in size and in number, in the pulpal blood vessels which were filled with red blood cells. On the labial side of the tooth, just beyond the outer enamel epithelial layer, large masses of red blood cells appeared. This response seemed similar to the red cell masses seen in cortisone-treated animals. It is possible that enough serum from these red cell masses could give the forming enamel a brown color. Beyond twenty injec-

tions, the effect was more severe and both ameloblasts and odontoblasts often lost their cytoplasm, with only the cell walls and ruptured nuclei remaining. Fig. 4 shows ameloblasts and odontoblasts after twenty-three injections. The weight of these animals increased in a normal curve. These rats averaged 160 Gm. at the start of the experiment and during the period of injections no animals were lost, except those killed intermittently, and there was an average weight increase of approximately 65 Gm. in each animal. Grossly, except for a slight loss of orange color by the time they had twenty injections, there was no apparent change in the incisor teeth. Microscopically, the appearance of enamel and dentin was altered. Degenerative changes in the salivary glands similar to those reported by Ogilvie[23] were found after twenty injections. A salivary gland from an animal after twenty-three injections is seen in Fig. 5, and a normal gland is seen in Fig. 6.

There was no apparent toxic effect on adult dba mice used for these experiments. Upon examination the vital organs did not differ grossly or microscopically from those in untreated animals. Adult dba mice did very well over periods of six months or longer. The teeth of newborn mice, offspring of animals treated with fluoride during the term of pregnancy, showed structural changes. There was retardation of calcification and a thickening of the ameloblasts' walls. It was evident that these concentrations of fluorides diffused through the placentas of mice. Similar findings were observed in the newborn and near-term guinea pigs.

Even after ninety days, where mice and guinea pigs were on 1:50,000 NaF drinking water, sections of enamel recovered from axillary and brain transplants were not hard and took a very deep hematoxylin stain. Further evidence of delayed calcification was shown by the failure of dentin to revise itself in transplants of long standing, as previously had been observed.[3, 5] When fluorides had been used, evidence for this attempted revision usually was lacking. Frequently a fibrocartilaginous or cartilaginous structure appeared in pulpal areas, often directly continuous with dentin formation. Figs. 9, 10, 11, 13, and 14 illustrate this. The first three figures show eye transplants and the latter two show brain transplants. Figs. 11, 13, and 14 show that cartilage will appear even when the tooth germs have been treated with methylcholanthrene. It thus seems that methylcholanthrene might have a latent effect as compared to fluorides. Fig. 12 shows the growth of a transplant in the anterior chamber after forty-six days. In this case there is no attempt at cartilage formation but, in places, a revision of the dentin into osteoid tissue has begun. Osteoid tissue,

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KEY TO SYMBOLS:

A—Ameloblast
B—Bone
Br—Brain
C—Cartilage
D—Dentin
E—Enamel
I—Iris
K—Keratin
Ly—Lymphocytes
N—Nuclei
O—Odontoblasts
P—Pulp
V—Vacuole
Art—Artifact
BV—Blood Vessel
CW—Cell Wall
DM—Descemet's Membrane
DT—Dentinal Tubules
ER—Enamel Rods
FiC—Fibrocartilage
KA—Kertinized Ameloblasts
Rbc—Red blood cells
SI—Stratum Intermedium
UD—Uncalcified Dentin
UE—Uncalcified Enamel

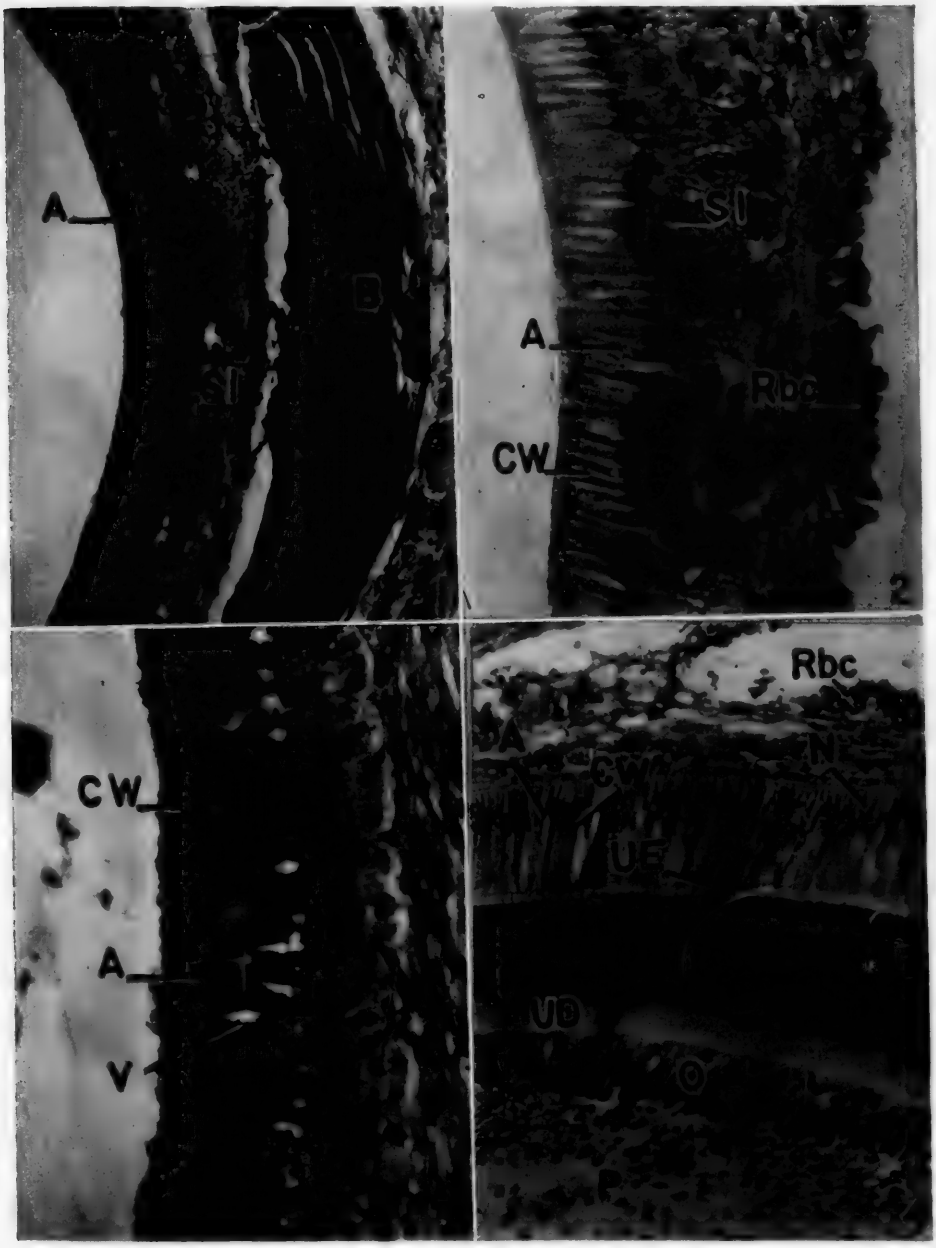


Fig. 1.—Normal ameloblasts in rat incisor. Section taken deep where ameloblasts are active. Original magnification, ×300; reduced ⅙.

Fig. 2.—Ameloblasts in rat incisor after ten injections of CaF_2. Note absence of some cells in stratum intermedium and accumulation of red blood cells. Original magnification, ×650; reduced ⅙.

Fig. 3.—Ameloblasts in rat incisor after fifteen injections of CaF_2. Note cuticular structure of ameloblasts. Original magnification, ×650; reduced ⅙.

Fig. 4.—Ameloblasts and odontoblasts in rat incisor after twenty-three injections of CaF_2. Note that odontoblasts are not as greatly affected. Original magnification, ×600; reduced ⅙.

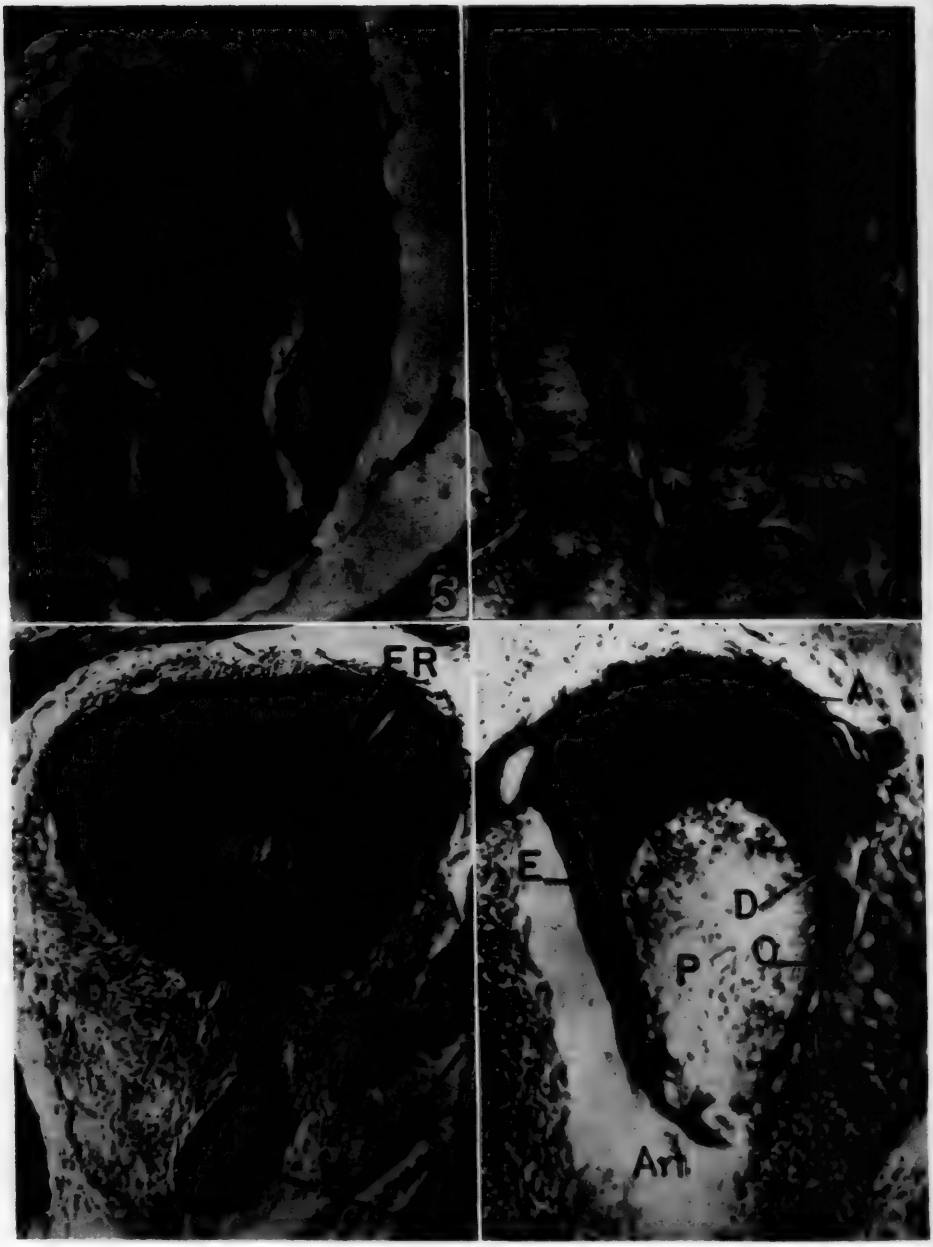


Fig. 5.—Secretory part of salivary gland in rat after twenty-three injections of CaF_2. Original magnification, ×300; reduced ⅙.

Fig. 6.—Secretory part of salivary gland in normal rat. Original magnification, ×300; reduced ⅙.

Fig. 7.—Cross section through axillary transplanted tooth germ in dba male mouse after thirty-six days. Tooth germ from 13-day embryo. Animal on 1:75,000 NaF. Note altered structure of enamel rods. Bone in center is not attached to transplant as seen in serial sections. Original magnification, ×250; reduced ⅙.

Fig. 8.—Cross section through axillary transplanted tooth germ in dba mouse after thirty-six days. Tooth germ from 13-day embryo. Original magnification, ×265; reduced ⅙.

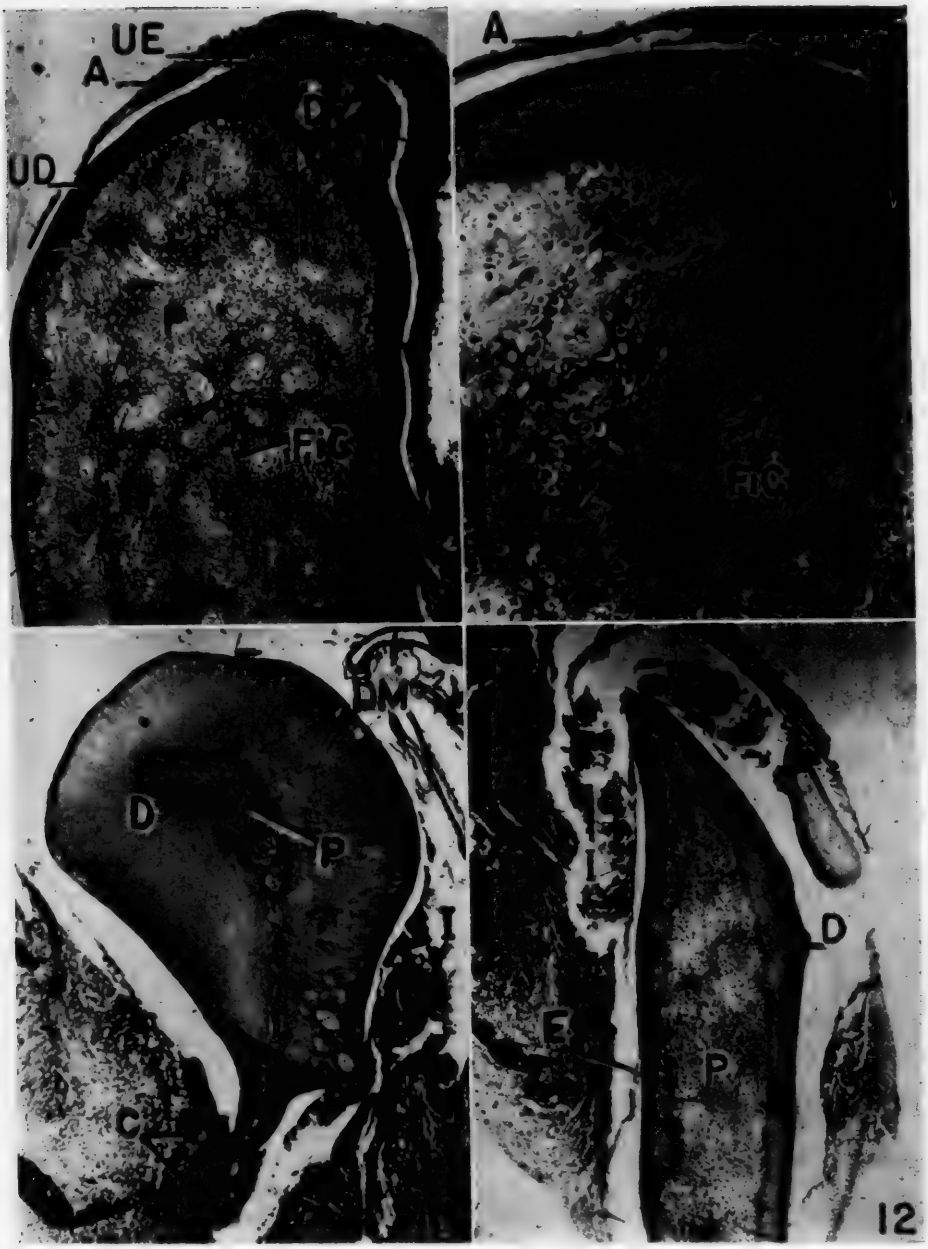


Fig. 9.—Section through recovered brain transplant of tooth germ in female guinea pig. Animal received seventeen injections of NaF and was killed after thirty-six days. Tooth germ from 23-day embryo. Note fibrocartilage structure in pulpal area. Original magnification, ×75; reduced ⅛.

Fig. 10.—Higher power of a through pulpal area to show structure. Original magnification, ×250; reduced ⅛.

Fig. 11.—Section recovered from male guinea pig's anterior chamber after forty-four days and twenty-five injections of NaF. Tooth germ from 22-day embryo. Note cartilage continuous with dentin formation at bottom of figure. Original magnification, ×60; reduced ⅛.

Fig. 12.—Transplant of tooth germ from 23-day embryo in anterior chamber of male guinea pig's eye after forty-six days. Original magnification, ×55; reduced ⅛.

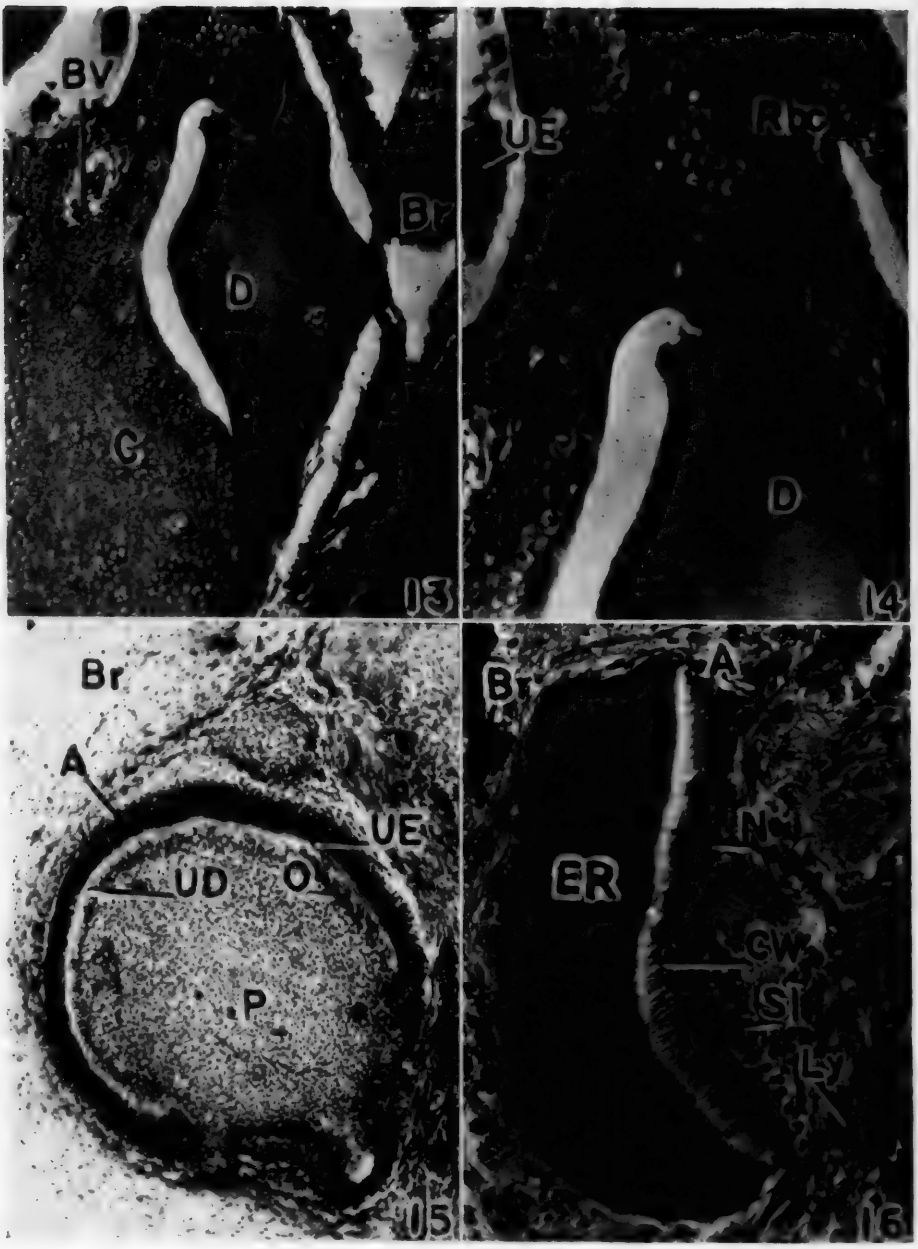


Fig. 13.—Transplant of tooth germs from 25-day embryo in male guinea pig's brain after forty-six days on oral CaF_2 1:50,000. Note cartilage continuous with dentin. Also note large blood vessels. Original magnification, ×150; reduced ⅙.

Fig. 14.—Higher power of Fig. 13 to show detail of cartilage and dentin. Original magnification, ×330; reduced ⅙.

Fig. 15.—Section from brain of male guinea pig after thirty-six days on 1:50,000 CaF_2. Tooth germ from 25-day embryo was impregnated with methylcholanthrene before transplanting. Note retardation of development and absence of keratin formation. Original magnification, ×165; reduced ⅙.

Fig. 16.—Section from brain of male guinea pig after tooth germ from 22-day embryo was impregnated with mythylcholanthrene. It was grown for forty-six days. Animal was on 1:50,000 NaF. Note structure of enamel (dark) and ameloblasts (light). Original magnification, ×160; reduced ⅙.

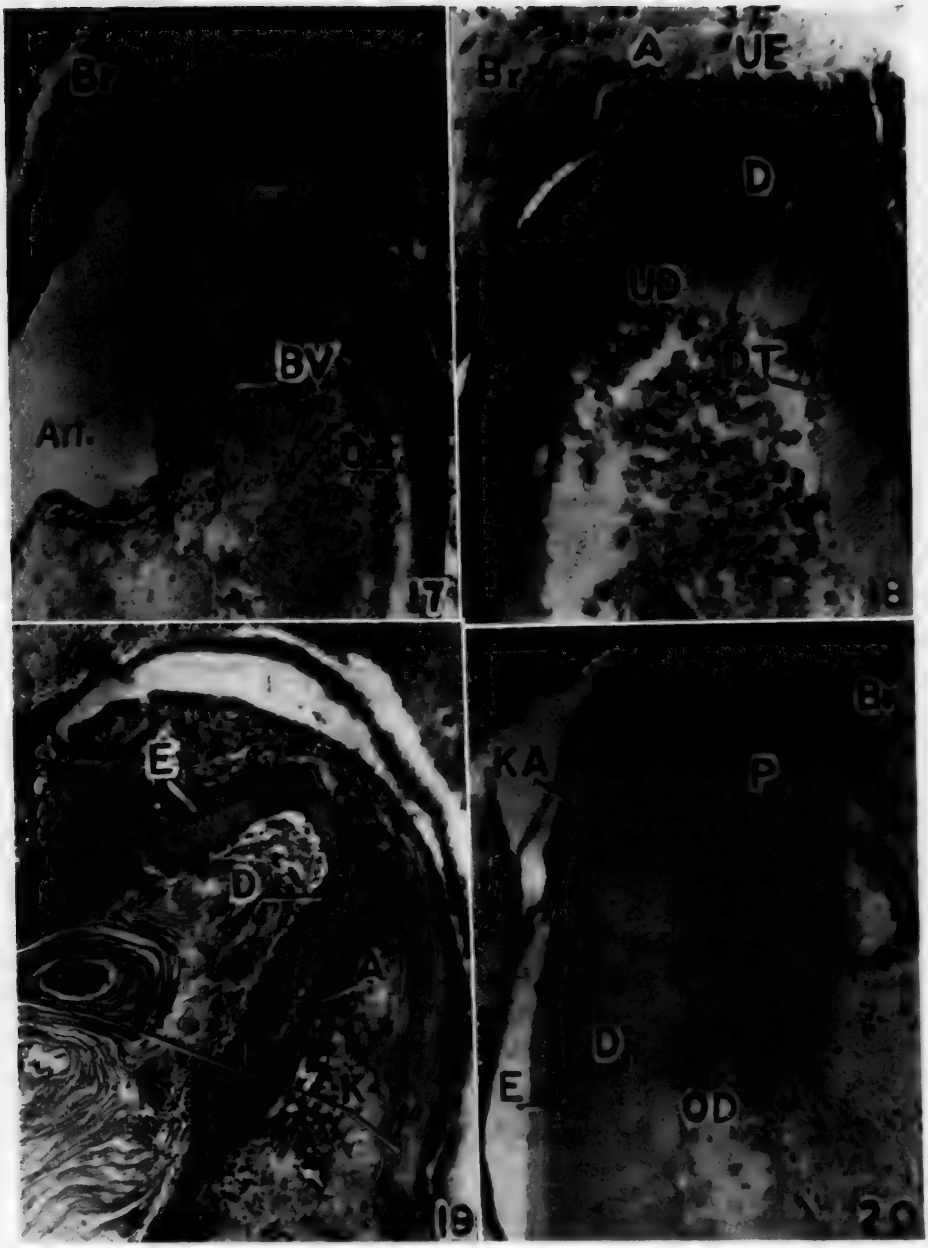


Fig. 17.—Section through brain transplant of tooth germ from 22-day embryo in female guinea pig. Tissue was impregnated with methylcholanthrene and animal was on 1:50,000 NaF for fifty days. Note lack of keratin and retardation of growth. Original magnification, ×155; reduced ⅙.

Fig. 18.—Higher power of deeper section of Fig. 17 to show alteration of ameloblasts. Original magnification, ×400; reduced ⅙.

Fig. 19.—Section recovered from female guinea pig's brain after forty-six days on 1:50,000 CaF_2. Tooth germ from 25-day embryo. Tissue was treated with methylcholanthrene. This shows expected effect of this chemical on transplanted tooth germs. Original magnification, ×260; reduced ⅙.

Fig. 20.—Section of recovered transplant in female guinea pig's brain after forty-six days. Tooth germ from 23-day embryo. This shows appearance of untreated transplant when animal was on regular drinking water. Original magnification, ×280; reduced ⅙.

when found in association with dentin formed in the transplants, assumed a faint stain. Bone frequently associated with these transplants of tooth germs (i.e., lying near and not joined with any part of the transplant) did not appear well calcified and often cartilage was also seen in these areas. Figs. 7, 16, and 18 show the alteration of enamel rods as formed in transplants in the presence of fluorides.

Lack of keratin formation in direct association with the transplanted tooth germ was the most significant observation. These observations were consistent even in the presence of methylcholanthrene. This chemical carcinogen affected adjacent tissues but not transplanted tooth germs. Further, it was observed that the major effect seemed to be on the ameloblasts and not the odontoblasts. Where the effect of methylcholanthrene on tooth germs had been observed previously, the cell walls of the ameloblasts stained pink.[4] However, in cases where fluoride was used these walls stained blue or purple. In the presence of methylcholanthrene, the walls of the ameloblasts appeared elastic and adhered to one another, but in the presence of fluoride they did not appear elastic, nor did they seem to possess any degree of adhesiveness for each other. They did, however, appear to retain their columnar or palisaded organization, which they did not retain in the presence of methylcholanthrene and in the absence of fluorides. Figs. 15 to 18 illustrate a lack of keratin formation with fluorides that usually appears, as seen in Fig. 19, in the presence of methylcholanthrene. Fig. 20 shows a brain transplant after thirty-six days where the tissue was not treated with methylcohlanthrene and the animal did not receive fluorides.

The comparative effect of the fluoride ion on transplanted embryonic lung tissue impregnated with methylcholanthrene was striking and is illustrated in Figs. 23 and 24. There was some intra-alveolar congestion after seventy-two days where the host was on drinking water with 1:50,000 NaF. However, in the absence of fluorides, methylcholanthrene appeared to have a greater effect on these embryonic lung transplants. In these cases there was greater congestion of the alveoli, and in many areas there was a piling up of epithelial cells. Some alveoli were filled with large squamous cells. In other alveoli epithelial pearls were formed, and in still other places there were large keratinous areas.

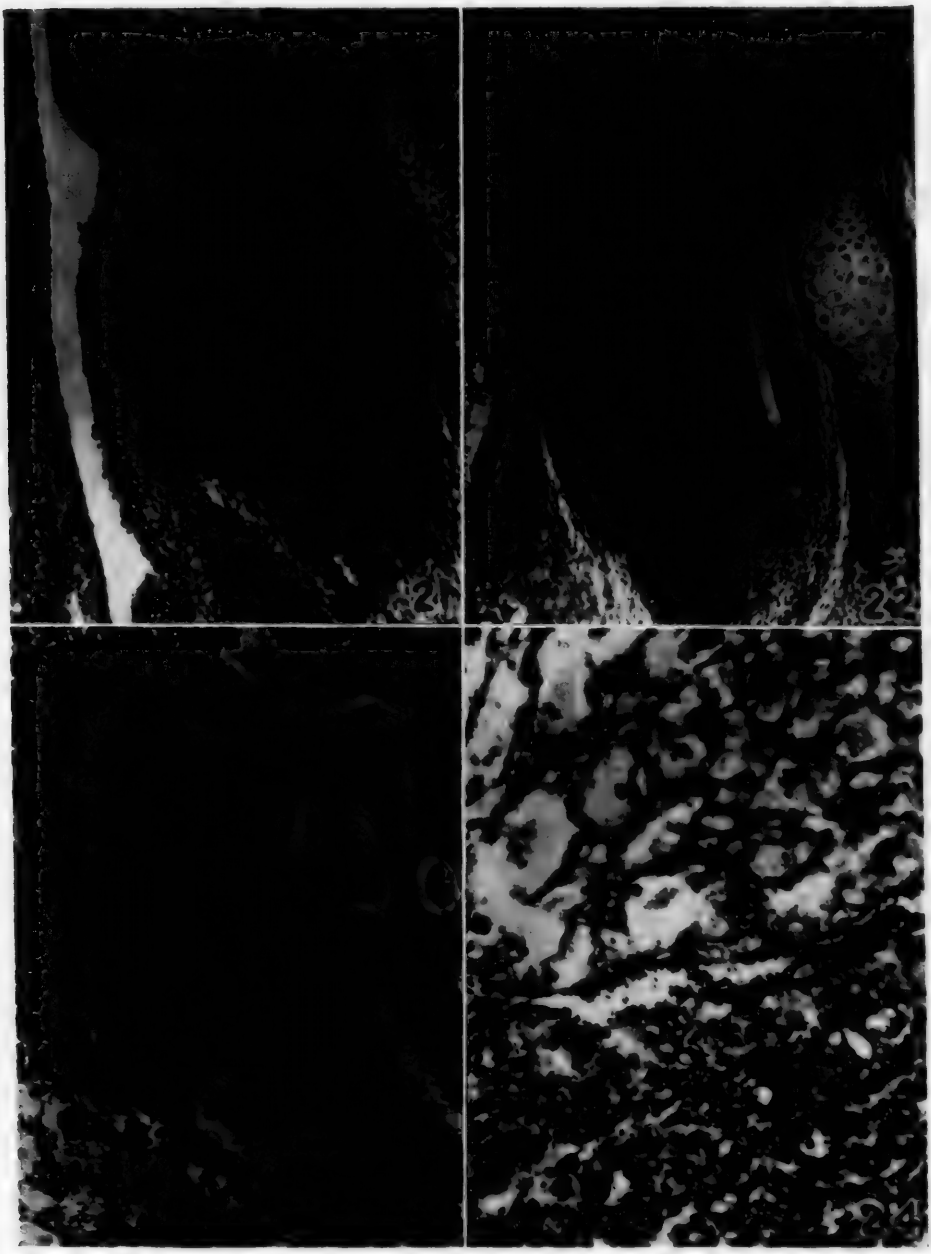
A striking observation was the consistent increase in lymphocytes about the recovered transplants. This was evident in all transplants, regardless of the site used for transplantation. Lymphocytes were near or adjacent to the transplants, which was not generally observed in the absence of fluoride administration. Also near by, in axillary and eye transplants, were areas of lymphatic tissue. There was, however, no actual round cell infiltration of the transplant. There also appeared to be an increased fertility of animals under fluoride administration.

Fig. 21.—Control section of mouse tooth germ at thirteen days. Original magnification, ×150; reduced ⅙.

Fig. 22.—Control section of guinea pig. Tooth germ from 23-day embryo. Original magnification, ×160; reduced ⅙.

Fig. 23.—Twelve-day embryonic dba lung treated with methylcholanthrene and transplanted to dba axilla after seventy-two days. Original magnification, ×290; reduced ⅙.

Fig. 24.—Twelve-day embryonic dba lung treated with methylcholanthrene and transplanted to dba axilla. Animal on 1:50,000 NaF. Section recovered after seventy-two days. Original magnification, ×290; reduced ⅙.



Figs. 21 to 24. *(For legends, see opposite page.)*

DISCUSSION

The effect of concentrations of fluorides as used in these experiments showed, among other things, retardation of expected calcification of enamel and dentin after formation in transplanted tooth germs. Significantly, in previous experiments very hard calcified structures were found over a comparatively short period.[3] In the presence of fluorides, however, a lack or retardation of calcification was evident in such transplants over much longer periods. Lantz and Smith,[14] Paff and Boyd,[24] and Goldenberg and Sobel[8] obtained similar results in their work with fluorides. However, the latter investigators, in their in vitro experiments, obtained calcification in the presence of the fluoride on ion when additional Mg + + was made available.

Three possible explanations may account for lack of calcification as observed in these experiments:

1. A deposition of calcium would not take place because its availability could have been interfered with by fluoride ions complexing with divalent ions, one of which may have been calcium. This explanation cannot be upheld because quantitatively there were not enough fluoride ions present under circumstances of these experiments to bind Ca + + or Mg + + present.

2. Lack of calcification may have resulted from interference with energy-producing systems at the cell level. This would be based on the fact that production of adenosine triphosphate was limited, due to interference with utilization of carbohydrates. As the carbohydrate system is interfered with, the production of adenosine triphosphate (ATP) is similarly blocked, resulting in a diminution of high-energy PO_4 bonds which must play an important part in mineralization.[15] It has been agreed that prior to mineralization the cells of the tooth germ contain glycogen which disappears as mineralization begins and subsequent sources of energy must be transported to these cells.[10, 11] All of these actions involve phosphorylation processes. Glock suggested that the phosphorylation processes involved in the breakdown of glycogen are the first source of the phosphate esters setting off this action.[7] According to Green,[9] the phenomenon, oxidative phosphorylation, has provided the most convincing and compelling evidence for the cyclophorase concept and step-wise of the many actions postulated by Krebs' citric acid cycle could be demonstrated in the cyclophorase system. Energy, therefore, must be available for the nucleus to form proenzymes which are necessary for the fate of the cell, and tying up one enzyme may alter the cell's fate. Green[9] also speaks of this interdependence of enzymes. Najjar[20] observed the mechanism of fluoride ions inhibition of enolase, suggesting the formation of Mg_2-fluoropho-enolase plus an Mg_2 salt. The prosthetic groups of enzymes concerned with carbohydrate utilization consist largely of known members of the vitamin B complex group such as riboflavin, nicotinamide, thiamine, and pyridoxine.[15] Inorganic ions act as activators for these enzyme systems, and Mg + + seems to be the principal ion concerned.[18] In addition, within the cell, activity of the fluoride ion seems to be at the enzyme level. Its action inhibits activity of the phosphatase enzyme, probably by complex formation with the enzyme itself.[15] Furthermore, enzyme

systems concerned with carbohydrate utilization are intimately associated with mitochondria.[9] This could account for alterations in the mitochondria of ameloblasts under influence of the fluoride ion as observed in these experiments. This slowing down of the total metabolic activity of ameloblasts in the present instance could result also in the lack of keratin formation as the end product in their life cycle.

3. The explanation also may be found at the cell membrane, involving systems concerned with transport of metabolites across this membrane in which the phosphatase enzyme is active. According to Albert,[1] architecture of cell walls, in the majority of cases, consists in the following order (extracellularly to intracellularly) of proteins, lipoids, and proteins in long flat molecules. Transport of sugars is by phosphorylation requiring energy-producing mechanisms to pump them into the cell; thus, enzyme systems must aid in this action.[1] Action of the fluoride ion may tie up enzyme transport systems in the cell walls, resulting in alteration of structure and permeability. If ionic transport across cell walls is interfered with, extracellular Ca + + is not available to the cell. Therefore, a shift in equilibrium can occur where calcium is resorbed from already formed enamel and dentin. Furthermore, to maintain equilibrium, Ca + + is shifted back to the cell; thus, preformed enamel and dentin does not calcify because additional Ca + + for calcification is not available. It also must be remembered that those cells concerned with the elaboration of enamel and dentin have an avidity for Ca_2 and $(PO_4)_3$. It is known, however, that the fluoride ion has an affinity for proteins, especially scleroproteins.[17] This, too, may contribute to the alteration of the permeability and structure of ameloblasts as observed in the present experiments. Since Ca + + for calcification may not be available, revision of dentin into osteoid tissue, as usually found when tooth germs are transplanted, is impossible.[5] Instead, sometimes cartilage is formed in association with dentin when fluorides are administered to animals bearing transplants of tooth germs.

Further support of the enzyme activity of the fluoride ion in the present experiments is substantiated by the fact that both CaF_2 and NaF, when used in like concentrations gave similar effects on tooth germ transplants. Since NaF is completely soluble and the solubility constant of CaF_2, as previously mentioned, is 0.0017 Gm. per 100 c.c. of H_2O at 26 degrees C., the action must have been at the enzyme level if their comparative solubilities are to be considered. Support of the action of fluorides at the cell wall is enhanced further by failure of ameloblasts to keratinize even in the presence of methylcholanthrene. From these observations it appears that the fluoride ion acts primarily at the cell wall, but it is also capable of diffusing across this structure and acting within the cell by tying up certain enzyme systems associated with the utilization of energy as derived from carbohydrates and stored in adenosine triphosphate. From these experiments, it appears that F^- ion is most effective when enormous amounts of energy are being consumed as pre-enamel and predentin are laid down. It thus appears that the fluoride ion in no case is directly concerned with calcification. Alterations as observed may be due to tying up enzyme systems concerned with phosphate transport which in turn mobilizes calcium, and

also to blocking the utilization of carbohydrates. The effect of fluoride on lung tissue that was impregnated with methylcholanthrene also substantiates the enzyme theory of action. At no time was there as much proliferation of cells when fluoride ions were present. The metabolic activity of these cells was slowed and epithelial pearls or keratin were not formed as in the absence of the fluoride ion. The increased lymphocytic activity would seem to indicate that the elaboration of cortisone-like substances from the adrenals was suppressed or anticortisone substances were increased. In view of the fact that the fertility of animals under fluoride administration appeared increased, the systemic activity of the fluoride ion possibly may have been at the pituitary level.

The effect of fluorides is primarily one that delays or slows down the mineralization process in developing transplanted teeth. It is reasonable, therefore, to conclude that there is more time for pre-enamel and predentin to be laid down before calcification begins. This may be an explanation of the increased bulk of pre-enamel and predentin as found in fluoride-treated animals, for the time before mineralization can begin obviously is delayed.

If the hypothesis can be accepted that fluorides affect the carbohydrate utilization in cells that have high energy requirements, it seems likely that this idea can apply to the control of neoplastic cells. It, therefore, seems possible that growth of fast-growing neoplasms may be retarded by the specific action of fluorides on the enzyme systems of these cells. This concept is being applied to control the growth of fast-growing spontaneous mouse tumors that have been retransplanted serially. It will be reported separately.

SUMMARY

1. Calcification of enamel and dentin was retarded in the presence of fluorides.

2. The structure of the ameloblasts was altered.

3. In the presence of methylcholanthrene and fluorides no keratinized cells of tooth germ epithelium were formed.

4. Dentin formed in transplants did not revise itself into osteoid tissue over long periods.

5. Cartilage frequently was found in association with dentin.

6. Action of the fluoride ion seemed at the enzyme level.

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A NEW APPROACH TO THE PROBLEM OF DENTAL CARIES CONTROL

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IT IS generally believed by most research workers that dental caries is caused by acids formed as a result of the action of microorganisms on carbohydrates. The process is characterized by a decalcification of the inorganic portion of the tooth and is accompanied by or followed by a disintegration of the organic matrix.[1] Theoretical considerations and some experimental data indicate that all of the carbohydrate that may be converted to acids for the production of dental caries is derived from the diet.[2] Furthermore, the only portion of the diet that may be effective in the caries process is that which is available on the tooth surface after the food is ingested. The acids are formed on the tooth surface, and, if they are not neutralized by the saliva or otherwise destroyed, the decalcification of the tooth will proceed.

On this basis it is obvious that in order to control dental caries one must prevent the acid from forming, produce conditions under which the acids will be destroyed before harm results, or make the tooth itself more resistant to the action of the acids. All of the methods that have been successful so far in the restriction of dental caries have conformed to one or more of these fundamental concepts. For example, the use of fluorides[3, 4, 5, 7, 8, 9] and soluble oxalates[6] have been shown either to make the tooth more resistant to acids or to have actually restricted dental caries. The elimination of fermentable carbohydrate from the diet[10-16] and increasing the natural neutralizing influences of the mouth have been partially successful in the restriction of dental caries.

In 1942 it was suggested that, in order to be effective in the control of dental caries, brushing of the teeth should be done immediately after eating. If the carbohydrates rapidly ferment on the tooth surface after the ingestion of sugars, it is clear that most of the damage from any particular ingestion is complete within a short interval of time. Thus, if the mouth were to be cleansed thoroughly immediately after the ingestion of the harmful ingredients, one should be able to eat sugars and still not be subject to dental caries. In 1942[17] a limited laboratory experiment indicated that caries could be controlled by oral hygiene procedures, but it was not until 1949[18] that a clinical investigation was carried out. The results of this experiment showed conclusively that dental caries was materially reduced by simply cleaning the mouth immediately after eating. The same effect has been produced in animals by not permitting the sugar to come in contact with the teeth.[19] Thus, when caries-active rats are given cariogenic diets by stomach tube, no carious lesions can develop.

From theoretical aspects of the problem, one of the most effective ways of preventing dental caries would be by the use of enzyme inhibitors. In so far as

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the acids which attack the tooth surface are derived from sugars by enzyme action, one should be able to block the formation of acid by the use of compounds that would inactivate the enzymes concerned. Such compounds as vitamin K, urea, ammonia, and a host of others are known to interrupt the chain of reactions which are necessary for the production of acids. In fact, there are literally hundreds of compounds of this nature which might be classified as enzyme inhibitors or antienzymes. The main difficulty involved, however, is a practical and effective method of application. It is obvious that, in order for the glycolytic reactions to be inhibited, the enzyme inhibitors must be present at the time the fermentable carbohydrate is present. This means that the inhibitor may be incorporated into the sugar that is ingested; it could be placed in the mouth immediately before, after, or during the consumption of sugar; or it may be fastened to the tooth surface in some manner so that it will be present when sugars are ingested. A compound suitable for incorporation in the sugar at the refinery would be highly desirable, but to date no such compound has been reported in the literature.

The introduction of enzyme inhibitors shortly before or after the ingestion of carbohydrates presents the same general problems as are entailed with the dietary control of dental caries and oral hygiene procedures. It necessitates a change in the usual habits of the individual, and, unless individuals can be interested sufficiently, the mass control of dental caries by these methods never will become popular.

On an experimental basis it was found that when vitamin K was placed into a chewing gum and used immediately after the ingestion of food there was an appreciable decrease in caries activity. It was found further that when calcium carbonate was placed into a chewing gum so that acids could be neutralized more readily there was also a decrease in caries activity. The main difficulties with this procedure were that it involved a change in the habits of the individual and, even though the procedure may control dental caries, it is not practical.

Ammonia, urea, and copper chlorophyllin have been incorporated into dentifrices on the basis that these ingredients would inhibit the fermentation of acids from carbohydrates. Only a 10 per cent decrease in caries activity was noted by the use of ammonia and urea.[20] There is no published clinical evidence that dentifrices containing the copper chlorophyllin are effective in reducing caries activity. These compounds are not retained on plaque material for an appreciable period of time[21] and are rapidly cleared from the mouth. Hence, they should not be particularly effective as enzyme inhibitors in this situation unless they can be placed in the mouth with each ingestion of sugar. Thus, some mechanism whereby the enzyme inhibitors may be retained on the tooth surface for longer periods of time is a prerequisite to the successful application of this method.

It has been noted by many investigators that the carious lesion always occurs under what is commonly called the dental or mucinous plaque. The dental plaque is consistently present on the unclean surfaces of the teeth. It is difficult to remove and by the usual oral hygiene procedures can be cleaned only

from those surfaces accessible to the toothbrush. It has been suggested many times that caries could be controlled if the dental plaque could be prevented from accumulating on the tooth surface, or if the dental plaque could be removed completely by means of oral hygiene procedures.

An investigation into the chemical composition and the physical and chemical characteristics of the plaque indicate that it is protein in nature[22-24] and is probably a natural protection against acid foods. It consists primarily of living and dead organisms and denatured salivary proteins. After it is completely removed it is rapidly redeposited so that, even though caries probably could be prevented by the elimination of the plaque, so far it has been impossible to keep the tooth free from plaque or even to prevent its formation. It is a source of most of the enzymes involved in the carious process and it serves as a sponge to hold the sugars and acids in contact with the tooth surface.

In 1949[25] it was suggested that if the dental plaque could not be eliminated or modified so as to be harmless it might serve as an agent for the retention of enzyme inhibitors on the tooth surface. The plaque is protein in nature and it is known that various stains will attach themselves to protein. This being the case, one might borrow the concept of chemotherapy from Ehrlich and incorporate an enzyme inhibitor into the molecule of a substance that would attach itself to the plaque. If compounds could be found that would absorb, adsorb, or otherwise attach themselves to the plaque, and at the same time be powerful enzyme inhibitors either in situ or by being gradually released from the plaque, these compounds could be incorporated into an effective therapeutic mouthwash or dentifrice.

Certain characteristics of the compound are desirable. In the first place, it must be effective in the prevention of acid formation from sugar, and it must attach itself to the plaque and should remain active for a period of several hours. It should be relatively nontoxic and it should be colorless. Furthermore, it should not be unpleasant to the taste and it should be readily available. With this concept in mind, screening technics were devised whereby the inhibition of acid formation and absorption onto the plaque surface could be determined. For the inhibition of acid formation the technics previously published were used, wherein the rate of acid formation in saliva-enamel-sugar mixtures was determined both with and without the inhibitor.[26-28] For the absorption or attachment onto the dental plaque the method of Fosdick, Ludwick, and Schantz was used,[21] or in some cases inhibitors were preliminarily screened by adsorption on casein. The casein then was rinsed well with water and placed in Snyder's medium with caries-active saliva.[29]

When it became apparent that compounds could be found that would become attached to dental plaques and remain after washing with water, it was of interest to determine whether or not the compounds would remain effective in the mouth. For this purpose the following technic was employed. Patients were selected from the student body and from those applying to the dental clinic for treatment. The pH of the plaque material from the proximal surfaces of noncarious teeth was measured according to the method of Campaigne.[30] The

mouth then was rinsed for thirty seconds with a 50 per cent sucrose solution, after which more plaque was removed from the same surface at five-minute intervals for twenty minutes, and the pH of each sample was measured. If, during the twenty-minute period, the acidity of the area was equivalent to a pH of 5.2 or below, the individual was considered suitable for the screening technic. It was found that only 33 per cent of the individuals had readily accessible areas of the teeth that satisfied this requirement.

When subjects were found that had readily available proximal tooth surfaces that consistently attained a pH value of 5.2 after the application of the sugar solution, they were issued a dentifrice or mouthwash containing an "active ingredient." They were instructed to either rinse their mouths or brush their teeth in the morning upon arising and in the evening before retiring for a period of seven days. On the seventh morning they returned to the laboratory for the sugar rinse. They were permitted to have breakfast, but they were not permitted to brush their teeth or to clean their mouths on the morning of the test.

The pH of the plaque material then was taken, after which the mouth was rinsed with a 50 per cent sugar solution and the acidity of the plaque was measured at five-minute intervals for twenty minutes as previously described. If the plaques did not attain an acid potential of 5.5 or below during the twenty-minute interval, the therapeutic agent was considered worthy of further study. Thus, if unmedicated plaques consistently formed acid to a potential of pH 5.2 or below when sugar was applied, and consistently did not attain a potential of pH 5.5 or below after one week of medication, the therapeutic was considered effective in regard to the inhibition of acid formation on the tooth surface. To date three compounds have been found to be effective in this manner, two of which are stable in water solution and in a dentifrice. Seven others show promise but have not been subjected to use in vivo.

RESULTS

Investigation of several hundred compounds indicated that, although most antiseptics, many aldehydes, ketones and anionic and cationic detergents, and other types of compounds, would prevent the formation of acid in sugar-saliva mixtures, very few would become attached to the plaque and remain active after washing with water.

The results of the screening test in regard to the inhibition of acid formation in sugar-saliva mixtures are shown in Table I. It should be noted that almost one-half of the compounds tested inhibited acid formation in a concentration of 50 mg. per 100 c.c. Furthermore, most of the antibiotics and antiseptics were effective in much smaller concentrations.

In contrast to the phenomenon of the inhibition of acid formation, of all the compounds tested, only penicillin, aureomycin, terramycin, subtilin, some amino acid derivatives of 5-nitrofuroic acid, sodium N-lauroyl sarcosinate,*

*The sodium N-lauroyl sarcosinate was synthesized in the Colgate-Palmolive-Peet laboratories and was found to be effective in the casein adsorption test. The adsorption on dental plaque was confirmed in this laboratory.

TABLE I
ACID INHIBITION IN SALIVA

A. COMPOUNDS EFFECTIVE AT 5 MG. PER 100 ML.

Acid acetone yeast extract, alkyldimethylbenzylammonium chloride, alkyldimethylethylbenzylammonium chloride, 4-amino-isophthalic acid, aureomycin, bacitracin, 1-(1-benzyl)-dodecylbenzyldimethylammonium chloride, bis-2-chloro-1,4-naphthoquinonyl-3-p-aminoazobenzene, calcium monofluorophosphate, cetyldimethylethylammonium bromide, cetyltrimethylammonium bromide, N4-(3-chloro-1,4-dihydro-1,4-dioxo-2-naphthyl)-N'-2-pyridylsulfanilamide, N4-(3-chloro-1,4-dihydro-1,4-dioxo-2-naphthyl)'-N-(2-pryrinidyl)-sulfanilamide, 2-chloro-3-(2,4-dimethylphenylamino)-1,4-naphthoquinone, 2-chloro-(3,4-dimethyl-2-pyridylamino)-1,4-naphthoquinone, 2-chloro-3-(β-hydroxyethylamino)-1,4-naphthoquinone, 2-chloro-3-(5-methyl-2-pyridylamino)-1,4-naphthoquinone, 2-chloro-3-morpholino-1,4-naphthoquinone, chloromycetin, 2-chloro-1,4-naphthoquinone, coco-dimethylamine · HCl, 2,6-di-(2-chloro-1,4-naphthoquinonyl)-aminopyridine, dihydrostreptomycin, N-dodecyl-β-alanine, dodecylaminolactate-dodecylamine-salicylate complex, n-heptylchlororesorcinol, (2-α-hydroxybenzyl)-1-dodecylbenzylammonium chloride, (2-α-hydroxybenzyl)-1-dodecylcinnamyldimethylammonium chloride, lauryldimethylamine · HCl, laurylisoquinolinium bromide, laurylphenylethyldimethylamine bromide, laurylphenylmethyldimethylamine chloride, 2,2'-methylenebis-(4-chlorophenol), 2,2'-methylenebis-(3,4,6-trichlorophenol), methylene blue, N-methyl-N-(5-nitrofuroyl)-p-aminophenol, N-methyl-N-(5-nitrofuroyl)-aniline, morpholinium ethyl sulfate, morpholinium soya ethyl sulfate, 5-nitrofuroic acid, N-(5-nitrofuroyl)-diethylamine, N-(5-nitrofuroyl)-ethanolamine, N-(5-nitrofuroyl)-phenylethanolamine, penicillin G, 1-(phenyl-1-decyl)-cinnamyldimethylammonium chloride, 1-phenyldodecylbenzyldimethylammonium chloride, 1-phenyldodecyldimethylcinnamylammonium chloride, soya trimethylammonium chloride, streptomycin, subtilin, 2-sulfanilamido-5-amyl-5-thiazolone, terramycin, p-tertiary-octylphenoxyethoxyethyldimethylbenzylammonium chloride, Zephiran chloride.

B. COMPOUNDS EFFECTIVE AT 10 MG. PER 100 ML.

Aceto-n-heptylresorcinol, 5-amino-5-methyl-1,3-bis-(2-ethylhexyl)-hexahydropyrimidine, aminopropyl tallow amine, cetyldimethylamine · HCl, cetyldimethylbenzylammonium chloride, N4-(3-chloro-1,4-dihydro-1,4-dioxo-2-naphthyl)-N'-guanylsulfanilamide, N4-(3-chloro-1,4-dihydro-1,4-dioxo-2-naphthyl)-N'-2-thiazolylsulfanilamide, 2-chloro-3-diisopropylamino-1,4-naphthoquinone, 2-chloro-3-(2-pyridylamino)-1,4-naphthoquinone, decylamine · HCl, di-n-butylresorcinol, dicaprylamine · HCl, dodecylamine lactate, dodecylamine undecylenate, formaldehyde, heptaldehyde, n-heptylresorcinol, hexadecylamine · HCl, n-hexylchlororesorcinol, lauryldimethylbenzylammonium chloride, myristyldimethylamine · HCl, 5-nitrofuramide, 5-nitrofuroylamide, N-(5-nitrofuroyl)-2-amino-3-methylpyridine, N-(5-nitrofuroyl)-6-amino-2-methylpyridine, N-(5-nitrofuroyl)-aniline, 5-nitrofuroylglycylglycine, N-(5-nitrofuroyl)-2-methyl-6-aminopyridine, N-(5-nitrofuroyl)-morpholine, N-(5-nitrofuroyl)-p-nitroaniline, octadecenyldimethylethylammonium bromide, octadecyldimethylbenzylammonium chloride, octylamine pectin, n-octyl chlororesorcinol, sodium N-myristoylsarcosinate, N-stearoylalanine, N-stearoylmorpholine, 2-sulfanilamido-4-methyl-5-chloropyrimidine, tallo amine · HCl, tetradecylamine · HCl, tyrothricin.

C. COMPOUNDS EFFECTIVE AT 25 MG. PER 100 ML.

Acetaldehyde, N-(acylcolaminoformyl)-methylpyridine chloride, 4-aminomethylbenzenesulfonamide · HCl, Armeen CD, cetyldimethylbenzylammoniumchlorobenzomercapto thiazole, cetyldimethylethylammonium chloride, 1-(3-chloro-1,4-dihydro-1,4-dioxo-2-naphthyl)-guanadine, hemicarbonate, Congo red, 2,3-dichloro-1,4-naphthoquinone, dodecylamine, ethyl-N-5-nitrofuroylaminocaproate, glutamic acid, 3-hydroxy-2-methylpyridine-1,4-naphthoquinone, isobutyraldehyde, 5-nitrofuroylaminobutanol, N-(5-nitrofuroyl)-2-amino-1-butanol, N-(5-nitrofuroyl)-2-amino-4-methylpyridine, N-(5-nitrofuroyl)-2-amino-5-methylpyridine, 5-nitrofuroylglycine, 5-nitrofuroyl-o-toluidine, octadecyldimethylethylammonium chloride, polymyxin, propionyl resorcinol, sodium N-lauroylsarcosinate, N-stearoylglutamic acid.

D. COMPOUNDS EFFECTIVE AT 50 MG. PER 100 ML.

N4-(3-chloro-1,4-dihydro-1,4-dioxo-2-naphthyl)-N'-(4-methyl-2-pyrimidyl)-sulfanilamide, N4-(3-chloro-1,4-dihydro-1,4-dioxo-2-naphthyl)-N'-sulfanilamide, 2-chloro-3-di-n-propylamino-1,4-naphthoquinone, 2-chloro-3-(3-methyl-2-pyridylamino)-1,4-naphthoquinone, N-(2-chloro-3-1,4-naphthoquinonyl)-glycine, N-(2-chloro-3-1,4-naphthoquinonyl)-lysine, dibenzylacetone, dimethylaniline picrate, enterogastrone, ethyl-N-(5-nitrofuroyl)-glycinate, gramicidin, 2-hendecynoic acid, 9-hendecynoic acid, isopropyl benzoate, laurylphenylpropyldimethylamine bromide, methyl-p-hydroxybenzoate, 2-methyl-3-hydroxy-1, 4-naphthoquinone, 2-methyl-1,4-naphthoquinone, α-naphthol-picrate, p-nitrobenzyl maleate, N-(5-nitrofuroyl)-δ-aminocaproic acid, N-(5-nitrofuroryl)-2-aminopyridine, 5-nitrofuroyldimethylamine, N-(5-nitrofuroyl)-diphenylamine, N-(5-nitrofuroyl)-ethylamine, 5-nitrofuroylglycylalanine, 5-nitrofuroylglycylglutamic acid, 5-nitro-

furoylglycylleucine, N-(5-nitrofuroyl)-glycine, N-(5-nitrofuroyl)-methylamine, 5-nitrofuroylmorpholine, n-propyl-p-hydroxybenzoate, pyocyanine dihydrochloride, secondary C_{16} and C_{18} amine acetate, tech., secondary coco amine · HCl, sodium N-(o-chlorobenzoyl)-sarcosinate, sodium-N-cinnamoylsarcosinate, sodium dehydroacetate, sodium p-naphthoquinone-4-sulfonate, sodium N-palmitoylsarcosinate, N-stearoyl-ethanolamine, N-stearoylglycylglycine N-stearoylmethionine, 2-sulfanilamido-5,5-diethyl-4-thiazolone, 2-sulfanilamido-4-dimethyl-5-bromopyrimidine, 2,4,6-tribromo-m-cresol, 2,4,6-trimorpholinomethylphenol.

E. COMPOUNDS NOT EFFECTIVE AT 50 MG. PER 100 ML.

Acetoguanamine, p-acetylaminobenzenesulfonyl-DL-alanine, p-acetylaminobenzenesulfonyl-glycine, p-acetylaminobenzenesulfonylglycyl-DL-phenylalanine, p-acetylaminobenzenesulfonyl-DL-isoleucine, p-acetylaminobenzenesulfonyl-DL-leucine, p-acetylaminobenzenesulfonyl-L-(-)-leucine, p-acetylaminobenzenesulfonyl-DL-norleucine, p-acetylaminobenzenesulfonyl-DL-norvaline, p-acetylaminobenzenesulfonyl-DL-phenylalanine, p-acetylaminobenzenesulfonyl-DL-valine, acetyl urea, DL-alanine, alanine · HCl, D-alanylglycine, Alperox C (technical lauroyl peroxide), p-aminobenzenesulfonyl-DL-alanine · HCl, p-aminobenzenesulfonyl-DL-isoleucine · HCl, p-aminobenzenesulfonyl-DL-leucine · HCl, p-aminobenzenesulfonyl-DL-norleucine · HCl, p-aminobenzenesulfonyl-DL-norvaline · HCl, p-aminobenzenesulfonyl-DL-phenylalanine, p-aminobenzenesulfonyl-DL-valine · HCl, 4-amino-3-pentadecylphenol · HCl, D-arabinose, L-arabinose, Benzedrine sulfate, benzoguanamine, N-benzoylsarcosine, S-benzylcysteine, beta methylumbelliferone, 2-bromo-3-decylamino-1, 4-naphthoquinone, N-(5-bromofuroic)-p-toluidine, N-(5-bromofuroyl)-glycylalanine, N-(5-bromofuroyl)-morpholine, p-bromophenacylfuroate, butyl-p-hydroxybenzoate, calcium indoleacetate, carbobenzoxy-D-alanine, carbobenzoxy-D-alanyl chloride, carbobenzoxy chloride, carbobenzoxyglycine, carbobenzoxyglycyl chloride, carbobenzoxyglycylglutamic acid, carbobenzoxyglycylglycine, carbobenzoxyglycylglycylglycine, carbobenzoxy-L-tryptophan, carbobenzoxy-L-tryptophanyl chloride, chloroacetyl chloride, chloral phenylhydrazone, 2-chloro-3-(2-aminoamyl)-1,4-naphthoquinone, 2-chloro-3-(ω-aminocaproic acid)-1,4-naphthoquinone, 2-chloro-3-decylamino-1,4-naphthoquinone, 2-chloro-3-di-sec-butylamino-1,4-naphthoquinone, 2-chloro-3-isobutylamino-1,4-naphthoquinone, 2-chloro-3-isopropylamino-1,4-naphthoquinone, 2-chloromethyl-5-hydroxy-1,4-pyrone, 2-chloro-3-propylamino-1,4-naphthoquinone, 2-chloro-3-sec-butylamino-1,4-naphthoquinone, N-cinnamoyl sarcosine, citrinin, N,N-dibenzoylcystine, N,N-dicarbobenzoxycystine, diketopiperazine, N,N-diphenylacetylcystine, diphenylacetyl-DL-ornithine, 2,5-ditertiarybutyl quinone, ethanolamine, ethyl-alpha-aminoisobutyrate, ethyl-N-benzoylsarcosinate, ethyl glycinate, ethyl glycinate · HCl, ethylidene glucose, ethyl-5-nitrofuroate, ethyl sarcosinate, ethyl sarcosinate · HCl, ethyl-N-stearoylglycylglycinate, ethyl-N-stearoylsarcosinate, ethyl vanillate, ferrous sulfate, formoguanamine, fumaryl glycidic acid, furfurylsemicarbazone, N-furoylsarcosine (anhydrous), glucosamine, L-glucose, glucosozone, D-glyceric aldehyde, DL-glyceric aldehyde, glyceryl-N-α-naphthylcarbamate, glycine, glycine anhydride, glycine · HCl, glycyl-L-glutamic acid, p-glycylaminobenzenesulfonyl-DL-phenylalanine · HCl, glycyl-alpha-aminoisobutyric acid, p-glycylaniline, glycylglycine, guanidinium mercaptocethane sulfonate, 10-hendecynoic acid, histamine, p-hydroxybenzoylalanine, p-hydroxybenzoylmethionine, p-hydroxybenzoylphenylalanine, p-hydroxyhippuric acid, 3-hydroxy-2-(methylacetone)-1,4-naphthoquinone, inositol, isoleucine, isoleucine · HCl, isopropyl-n-phenylcarbamate, leucine, leucine · HCl, maleic anilide, mannuronic acid lactone, Marsilid phosphate, 3-mercapto-1-trimethylammonium bromide propane, 2-methoxy-1,4-naphthoquinone, methylaminoacetonitrile, 2-methyl-3-hydroxynaphthalene, 2-methyl-3-methylacetate-1,4-naphthoquinone, morpholine, N-(p-Nitrobenzoyl)-sarcosine, p-nitrobenzylacetyl salicylate, N-5-nitrofuroylalanyltyrosine, 5-nitrofuroylglycyllysine, 5-nitrofuroylglycylphenylalanine, 5-nitrofuroyl-m-nitroaniline, 5-nitrofuroyl-p-nitroanaline, N-(5-nitrofuroyl)-p-toluidine, N-(3-nitrophthalyl)-2-sarcosine, 2-nonone-3-hydroxy-1,4-naphthoquinone, norleucine, norleucine · HCl, norvaline, norvaline · HCl, Palatone, D-pantothenyl alcohol, phenacylacetylsalicylate, phenolphthalein, phenylacetyl-L-alanine, phenylacetyl-L-arginine, phenylacetyl-L-cystine, phenylacetylglycine, phenylacetylglycylvaline, phenylacetyl-L-lysine, phenylacetyltryptophan, D,L-phenylalanine, phenylalanine · HCl, picric acid, pontamine white dye, n-propoxy-2-amino-4-nitrobenzene, pyrogallol triacetate, salicylaldehyde semicarbazone, sodium benzoate, sodium N-benzoylsarcosinate, sodium bisulfite, sodium borate, sodium N-(p-chlorobenzoyl)-sarcosinate, sodium N-diphenylacetylsarcosinate, sodium N-(p-ethoxybenzoyl)-sarcosinate, sodium N-furoylsarcosinate, sodium p-hydroxybenzoylaspartate, sodium p-hydroxybenzoylglutamate, sodium p-hydroxybenzoylisoleucinate, sodium p-hydroxybenzoylnorleucinate, sodium p-hydroxybenzoyltryptophanate, sodium p-hydroxybenzoylvalinate, sodium N-(o-iodobenzoyl)-sarcosinate, sodium N-(p-nitrobenzoyl)-sarcosinate, sodium 3-nitrophthalylalaninate, sodium 3-nitrophthalylaspartate, sodium 3-nitrophthalyl-L-glutamate, sodium 3-nitrophthalylglycinate, sodium 3-nitrophthalyl-DL-isoleucinate, sodium 3-nitrophthalyl-DL-leucinate, sodium 3-nitrophthalylmethionate, sodium 3-nitrophthalylphenylalanate, sodium 3-nitrophthalylvalinate, sodium phthalylglycylglycinate, sodium N-stearoylsarcosinate, sodium N-(m-toluoyl) sarcosinate, sodium N-(o-toluoyl)-sarcosinate, sodium N-(p-toluoyl)-sarcosinate, N-stearoylalanyl hydrazide, N-stearoyl-δ-aminocaproic acid, N-stearoyl-δ-aminocaproic ester, N-stearoylglycine, N-stearoylglycyl hydrazide, N-stearoylsarcosine, N-

stearoyltaurine, 2-sulfanilamido-5-bromopyrimidine, 2-sulfanilamido-5-chloropyrimidine, 2-sulfanilamido-4,6-dimethyl-5-chloropyrimidine, 2-sulfanilamido-5,5-dimethylthiazolone, 2-sulfanilamido-5-ethyl-4-thiazolone, 2-sulfanilamido-4-methyl-5-bromopyridine, 2-sulfanilamido-4-thiazolone, tannic acid, taurine, threonine, N-(m-toluoyl)-sarcosine, N-(o-toluoyl)-sarcosine, N-(p-toluoyl)-sarcosine, L-tryptophanylglycine, tyrocidine, valine, valine . HCl, D-xylose, L-xylose.

sodium dehydroacetate,* sodium N-myristoyl sarcosinate, (1-phenyl-1-decyl)-cinnamyl-dimethyl-ammonium chloride and 1-phenyldodecyl-benzyl-dimethyl-ammonium chloride were retained effectively on the plaque after the washing period. It also should be noted that there seems to be no relation between the activity of the acid inhibitor and retention on the plaque.

It is obvious that before a therapeutic ingredient may be used in vivo on human beings it must be proved harmless to the individual. If it is to be used in a dentifrice or mouthwash it must be compatible with the type of preparation, it must be stable over relatively long periods of time, and it must be pleasing to the taste and sufficiently active so that it may be incorporated in reasonable quantities. Furthermore, it is quite probable that compounds widely used in medicine, such as many of the antibiotics, should not be used in a dentifrice or mouthwash, even though they may be effective in preventing dental caries. If the use of an antibiotic in a dentifrice reduced the effectiveness of the antibiotic in serious diseases, then its incorporation into dentifrices or mouthwashes would be contraindicated.

Of all of the compounds that were found to be effective in the prevention of acid formation, and that at the same time resisted removal from the plaque material, only two are known to fulfill all of the above requirements. Sodium N-lauroyl sarcosinate and sodium dehydroacetate are nontoxic, they are stable and can be incorporated effectively into a dentifrice or mouthwash, they prevent the acid formation in saliva-sugar mixtures, and they are retained effectively on plaque material. None of the derivatives of 5-nitrofuroic acid, nor any of the effective detergents other than sodium N-lauroyl sarcosinate, have been studied sufficiently in regard to toxicity for in vivo studies. All of the other compounds that satisfied the above requirements are antibiotics; however, many of these are unstable in aqueous solution. For this reason, at least for the present, only sodium N-lauroyl sarcosinate and sodium dehydroacetate were selected for in vivo testing in dentifrices or mouthwashes.

Inasmuch as penicillin has been investigated intensively in regard to the prevention of dental caries, and inasmuch as the sodium dehydroacetate and the sodium N-lauroyl sarcosinate behave very similarly to penicillin in regard to the prevention of acid formation in saliva mixtures and in regard to retention on plaque material, it was decided to include penicillin in a tooth powder as a reference material. It was found that, after seven days' use in a therapeutic dentifrice or mouthwash, all three of these compounds would prevent the tooth surface of individuals using the therapeutic from becoming more acid than pH 5.5. Fig. 1 shows the relative effectiveness of these compounds. As can be seen from this graph, the maximum acidity obtained after sucrose was the same

*The sodium dehydroacetate was shown initially to be effective for acid inhibitory activity and adsorption on plaque in vivo in the laboratories of the Lambert Pharmacal Company Division of the Lambert Company.

for all three compounds. Penicillin was tested on sixty individuals, sodium N-lauroyl sarcosinate was tested on 144 individuals, and sodium dehydroacetate was tested on 145 individuals.

In addition to the above, other tests were performed in order to determine whether or not continued use of the potentially therapeutic compounds would build up a tolerance, such as has been observed with penicillin.[33] Large scale clinical experiments were started wherein the subjects used the therapeutic products in the morning upon arising and in the evening before retiring. Adequate placebo and control groups also were included. Complete dental examinations, including radiographs, were made in order to determine the number of carious lesions present. It was intended that these experiments would continue for a period of two years, after which time the clinical efficacy could be determined.

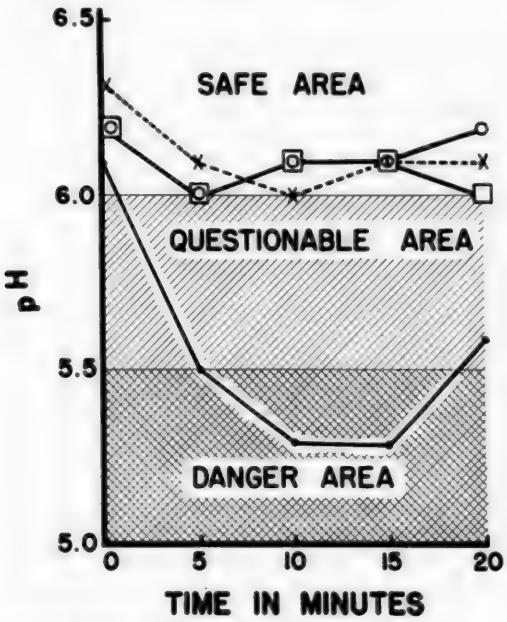

Fig. 1.—Average plaque pH after sucrose rinse.
• Control
□ Penicillin
O Sodium N-lauroyl sarcosinate
X Sodium dehydroacetate

At the end of a nine-month period, the acidity attained on the tooth surface after a 50 per cent sugar rinse was determined in the same manner as described in the seven-day test.

It was found (Table II) that 44 per cent, 95 per cent, and 89 per cent of the cases remained above pH 5.5 during the twenty-minute interval after the use of the placebo, sodium lauroyl sarcosinate, and sodium dehydroacetate, respectively. Thus, it would seem that both of the compounds remained active after the nine months' use.

TABLE II
ACID FORMATION ON THE TOOTH SURFACE AFTER NINE MONTHS' USE OF THERAPEUTIC DENTIFRICE

	PLACEBO		SODIUM LAUROYL SARCOSINATE		SODIUM DEHYDROACETATE	
pH	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
5.2 and below	65	56	4	5	6	11
5.3 to 5.5	99		9		4	
5.6 to 5.9	98	44	28	95	32	89
6.0 and above	30		215		50	
Totals	292		256		92	

DISCUSSION

If dental caries is initiated by a characteristic decalcification by acids held in close contact with the tooth surface by means of the dental plaque, and if the acids in turn are formed by the microbial enzymatic degradation of fermentable carbohydrates, it should be possible to prevent dental caries by the proper use of enzyme inhibitors. The use of the ammonium ion did not give entirely satisfactory results, and may be explained on the basis that most substances, including the ammonium ion and ammonia, are cleared rapidly from the mouth by the salivary flow. We now have substances that will impede or prevent the formation of acids from fermentable carbohydrates and at the same time will attach themselves to the dental plaque for relatively long periods of time. Thus, the rate of acid formation is decreased materially, even in the presence of sugars, for long periods of time, or sugars may be taken into the mouth at any time during this interval with no harmful results.

There have been two experiments in which an inhibitor (penicillin) that is retained by the dental plaque has been used in a dentifrice for the control of dental caries. In the first instance, there was a marked decrease in caries activity as determined by clinical means. The results of the experiment by Zander[31] are very convincing. Another experiment by Hill and Kniesner[32] reported no decrease in caries activity, but the failure was attributed to the difficulty in obtaining cooperation of the experimental subjects. It was shown conclusively that many of the subjects did not use the dentifrice consistently.

It is known that penicillin will prevent the formation of acids from sugar. It is known further that this substance will be retained on the dental plaque for from twelve to twenty-four hours. There is excellent evidence that the plaques in situ that contain the penicillin will resist changes in pH after the application of sugar, and there is excellent evidence that when a penicillin dentifrice is used consistently there is a marked decrease in caries activity. In view of this, it is quite logical that other substances that perform in the same manner should cause a material decrease in caries activity when used in a dentifrice or mouthwash.

To date about ten compounds have been found which will become attached to the plaque material in effective concentrations and resist washing with water.

Of these, three have been incorporated into dentifrices or mouthwashes and have been tested in vivo. In each case the results have been favorable. Two of these, sodium N-lauroyl sarcosinate and sodium dehydroacetate, are now under test clinically. After nine months of this clinical test, it was found that both compounds were still effective in blocking acid formation below what has been considered the harmful level. This dividing line is empirical and is based primarily on laboratory evidence. No accurate estimates concerning the effect of these compounds on clinical caries can be made, but it would be interesting to speculate on the possible results. If the dangerous acidity is pH 5.5 and the carious process is an all-or-none proposition, then there should be no appreciable differences in the caries-inhibiting action of the two compounds. However, if this is not the case, they might possibly develop a difference, as one of the compounds depressed the formation of acid to a greater extent.

If we assume that acid on the tooth surface is the immediate cause of dental caries, then dentifrices or mouthwashes containing sodium N-lauroyl sarcosinate, sodium dehydroacetate, penicillin, and other compounds which behave in the same manner should be truly therapeutic in the prevention of dental caries.

SUMMARY

A new approach to the problem of dental caries control based upon the retention of acid inhibitors in dental plaque has been suggested. Laboratory technics for the screening of compounds to conform to this theory were devised and performed on 381 compounds. Of these compounds, ten were found to be effective in the screening technics. Three of these were tested in vivo in dentifrices or mouthwashes and it was found that all three materially decreased the rate of acid formation on the tooth surface over long periods of time. Continued daily use of these compounds has shown that their efficiency is maintained. Two of these compounds, sodium N-lauroyl sarcosinate and sodium dehydroacetate, are now under clinical test to measure quantitatively their therapeutic effect on dental caries.

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SODIUM AND POTASSIUM AS CONSTITUENTS OF HUMAN SALIVARY BUFFERS

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THE saliva of human beings is so well buffered that it maintains a rather constant pH despite the introduction into the mouth of substances containing high concentrations of acids or alkalies. Even when the pH of saliva is altered by such means, the changes are of comparatively short duration. The stimulation of the salivary glands arising from mastication results in an increased flow which acts as a diluent. The maintenance of the homeostatic character of the salivary pH in the presence of acidic or basic foods and liquids is further facilitated by an increase in the buffer capacity of the saliva secreted in response to stimulation. This effect was first observed by Marshall,[1] who found that the use of paraffin as a salivary stimulant yielded a saliva whose neutralizing power was considerably greater than that collected from the same patients in the basal state. Marshall's observations have since been confirmed by numerous investigators.[2, 3]

In recent years, considerable attention has been devoted to the elucidation of the anionic components of the buffer pairs of human saliva. Wah Leung[4] showed that stimulated saliva at or near the pH of that freshly collected from the mouth owes its buffering power primarily to bicarbonates with minor contributions from the contained phosphates and proteins. Sellman[5] observed that at physiologic pH levels, the bicarbonates and phosphates are the principal buffers of saliva, with the bicarbonates assuming a preponderant role in paraffin-activated saliva. The cationic concentrations of the salivary buffer systems remain, however, a problem for investigation.

Quantitatively, sodium, potassium, calcium, and magnesium are the most important basic constituents of tissues and body fluids. While each of these cations is present in human saliva, the insolubility of the calcium and magnesium salts of carbonate, phosphate, and protein under physiologic conditions precludes their contributing to the buffering power of saliva. Because of the high solubility of the salts of sodium and potassium, it was postulated that the level of these elements may be a factor limiting the buffering capacity of saliva. Accordingly, the present study was undertaken to ascertain (1) whether the sodium and potassium concentrations of saliva are related to its buffer capacity, and (2) the degree to which any relationship between the buffer capacity and sodium and potassium content of saliva is influenced by its rate of flow.

MATERIALS AND METHODS

The rate of flow, buffer capacity, and sodium and potassium levels of saliva were determined on samples collected from eighty consecutive patients seen at

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the Nutrition Clinic of the Hillman Hospital, Birmingham, Ala. Paraffin-stimulated saliva obtained between 8 A.M. and 10 A.M. was used for these analyses. The paraffin blocks which served as secretory stimulants were completely free from detectable amounts of sodium and potassium. The rate of flow in response to mechanical stimulation was established by pooling all saliva secreted by each patient during a thirty-minute interval in graduated cylinders containing a layer of paraffin oil. Readings were recorded to the nearest milliliter. The buffer capacity was determined on a 5 ml. aliquot obtained by inserting a graduated pipette below the layer of oil and drawing up and down several times to secure a representative sample. The method used to measure the buffer capacity was that developed by Dreizen, Mann, Cline, and Spies.[6] Oil-free Pyrex tubes were used to collect the samples subjected to analysis for sodium and potassium. Sodium and potassium concentrations were determined with a Perkin-Elmer Model 52-A flame photometer by the procedure of Bills, McDonald, Niedermeier, and Schwartz[7] based on the internal standard technic of Berry, Chappel, and Barnes.[8]

Samples of unstimulated and paraffin-stimulated saliva provided by each of twenty-five additional patients were used to establish the differences in buffering capacity and in the concentrations of the anions and cations comprising the buffer systems of resting and activated saliva. All samples were tested for buffer capacity, bicarbonate, phosphate, protein, sodium, and potassium content. The concentrations of bicarbonate, phosphate, and protein were determined by the methods of Van Slyke and Neill,[9] Fiske and Subbarow,[10] and Wolfson, Cohn, Calvary, and Ichiba,[11] respectively.

OBSERVATIONS

The Relationship of the Buffer Capacity of Stimulated Saliva to the Rate of Flow and to the Sodium and Potassium Content.—As can be seen from Table I, in which the eighty samples have been grouped according to volume, the buffer capacity of the saliva was, in general, directly related to the rate of flow (Fig. 1). The mean buffer content ranged from an amount equivalent to 0.215 ml. 0.1 N lactic acid per 5 ml. saliva for the group containing from 1 to 20 ml. of saliva to an equivalent of 0.825 ml. 0.1 N lactic acid per 5 ml. saliva for the group containing from 101 to 120 ml. A similar relationship was noted for the mean sodium content of each group of saliva samples (Fig. 2). Thus, the mean

TABLE I
COMPARISON OF THE RATE OF FLOW, BUFFER CAPACITY, AND SODIUM AND POTASSIUM LEVELS OF 80 SAMPLES OF PARAFFIN-STIMULATED WHOLE SALIVA

RATE OF FLOW (ML. PER 30 MINUTES)	NUMBER OF SAMPLES	BUFFER CAPACITY (ML. 0.1 N LACTIC ACID PER 5 ML. SALIVA)		SODIUM (MEQ. PER L.)		POTASSIUM (MEQ. PER L.)	
		MEAN	RANGE	MEAN	RANGE	MEAN	RANGE
1 to 20	4	0.215	0.17 to 0.25	14.8	6.5 to 21.7	21.2	19.0 to 23.3
21 to 40	32	0.482	0.16 to 0.90	19.3	6.1 to 41.3	18.8	11.3 to 25.6
41 to 60	25	0.601	0.32 to 0.88	23.2	10.9 to 40.9	19.0	14.9 to 25.6
61 to 80	15	0.656	0.40 to 0.93	32.2	14.8 to 51.7	19.5	12.8 to 24.9
81 to 100	2	0.840	0.83 to 0.85	38.0	34.8 to 41.3	18.3	15.6 to 21.5
101 to 120	2	0.875	0.85 to 1.10	44.6	43.0 to 46.1	18.3	17.9 to 18.7

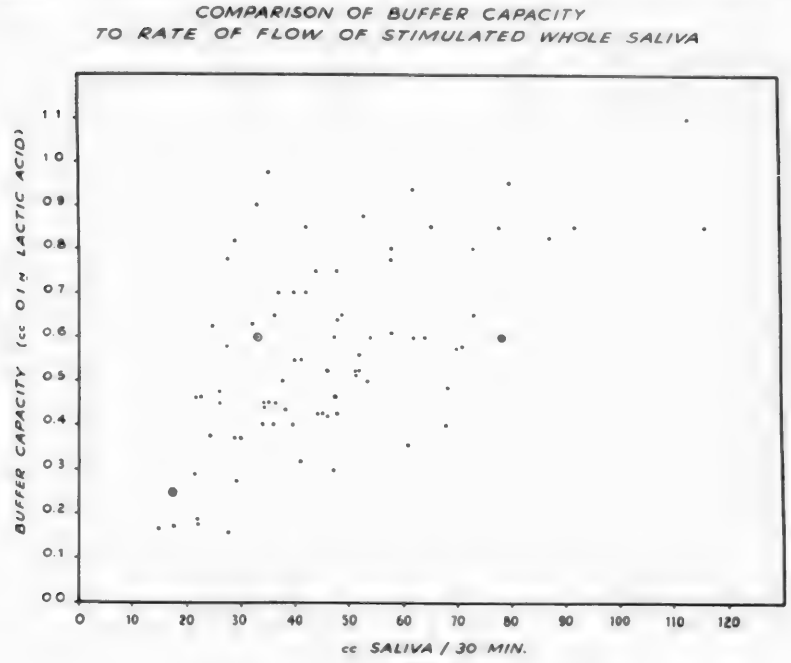
COMPARISON OF BUFFER CAPACITY
TO RATE OF FLOW OF STIMULATED WHOLE SALIVA
BUFFER CAPACITY (cc 0.1 N LACTIC ACID)
cc SALIVA / 30 MIN.

Fig. 1.

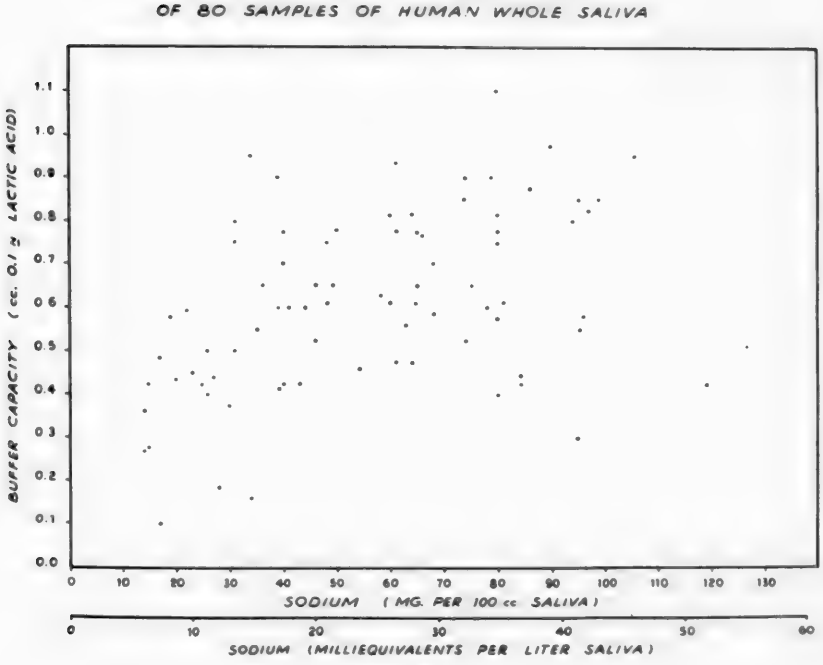
BUFFER CAPACITY AND SODIUM CONTENT
OF 80 SAMPLES OF HUMAN WHOLE SALIVA
BUFFER CAPACITY (cc. 0.1 N LACTIC ACID)
SODIUM (MG. PER 100 cc SALIVA)
SODIUM (MILLIEQUIVALENTS PER LITER SALIVA)

Fig. 2.

sodium values extended from 14.8 millieqivalents per liter for the group containing 1 to 20 ml. of saliva to 44.6 millieqivalents per liter for the group containing 101 to 120 ml. In contrast, the mean concentrations of potassium were of the same order of magnitude for each group of samples regardless of volume or buffer capacity (Fig. 3). While the buffer capacities and sodium content of the eighty saliva samples were directly related to volume when the samples were grouped on the basis of the amount of saliva secreted during the thirty-minute period of collection, the values for individual samples showed some deviation. When these data were subjected to statistical analysis, the following correlation coefficients* were obtained: buffer capacity and rate of flow, 0.69; buffer capacity and sodium level, 0.49; buffer capacity and potassium level, –0.08. The standard errors of correlation* were 0.059, 0.083, and 0.11, respectively. A correlation coefficient three or more times its standard error is considered to be statistically significant. Applying this formulation, it will be noted that the relationship between buffer capacity and rate of flow and between buffer capacity and sodium content were significant, whereas that between buffer capacity and potassium content was not significant.

The Anionic and Cationic Content of the Buffer Systems of Resting and Paraffin-Activated Saliva.—The partition of the anions and cations comprising the salivary buffers is shown in Table II. The buffer capacity and the bicarbonate and sodium concentrations of the samples of paraffin-stimulated saliva exceeded those of resting saliva in each of the twenty-five patients tested. In almost all instances, the differences were pronounced. In contrast, the phosphate and protein content of unstimulated saliva exceeded that of activated saliva in twenty-one and nineteen of the twenty-five patients, respectively; whereas the differences between the potassium concentration of the two types of saliva obtained from each patient were not remarkable. While the potassium concentration was greater than the sodium content in all but two of the twenty-five samples of resting saliva, the reverse relationship was observed in twenty-two of the twenty-five samples of paraffin-stimulated saliva. Similarly, the phosphate concentration exceeded the bicarbonate concentration in all but one of the twenty-five samples of resting saliva, whereas the bicarbonate concentration was greater than that of the phosphate in all samples of stimulated saliva. In every instance, the combined amount of sodium and potassium present in the specimens

*Coefficient of correlation (r):

$$r = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}}{\sqrt{\left(\Sigma x^2 - \frac{(\Sigma x)^2}{N}\right)\left(\Sigma y^2 - \frac{(\Sigma y)^2}{N}\right)}}$$

x = buffer capacity
y = rate of flow or sodium content or potassium content
N = number of samples

Standard error of the coefficient of correlation (σr):

$$\sigma r = \frac{1 - r^2}{\sqrt{N}}$$

r = coefficient of correlation
N = number of samples

of stimulated saliva was substantially greater than the total amount of these elements present in the samples of resting saliva obtained from the same patient under similar conditions.

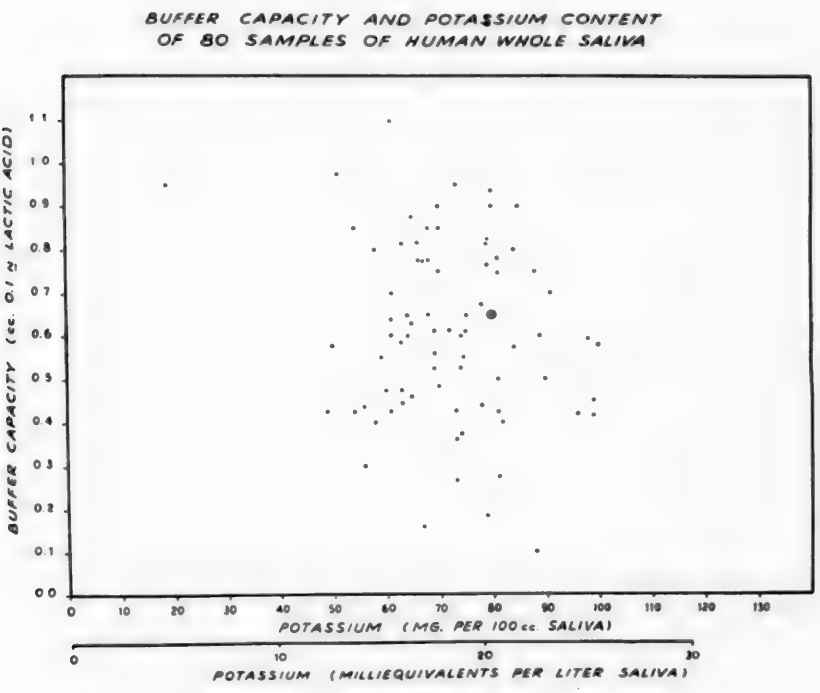

Fig. 3.

DISCUSSION

The present findings demonstrate that the buffer capacity of stimulated saliva is considerably greater than that of resting saliva in the same person. In this series, the increases in the buffering power of stimulated saliva as contrasted to that of resting saliva ranged from 27 per cent to 400 per cent, with a mean of 150 per cent. As seen in Fig. 1, the magnitude of difference was, in general, related to the rate of flow.

The difference in the buffer capacities of resting and stimulated saliva in the same person is related to the sodium and bicarbonate concentration. Whereas potassium is the predominant cation in the buffer systems of resting saliva, sodium predominates in stimulated saliva. Of the anions contributing to the salivary buffers, phosphates and proteins decrease in concentration and bicarbonates increase in concentration when the salivary glands are activated by the masticatory process. In twenty-two of twenty-five cases studied, the total increase in sodium and bicarbonate concentration of stimulated saliva was considerably greater than the total change in potassium, phosphate, and protein

TABLE II
THE ANIONIC AND CATIONIC CONTENT OF THE BUFFER SYSTEMS OF RESTING AND PARAFFIN-STIMULATED HUMAN SALIVA

PATIENT	BUFFER CAPACITY (ML. 0.1 N LACTIC ACID PER 5 ML. SALIVA)		BICARBONATE (HCO_3^-)* (MEQ. PER L.)		PHOSPHATE ($HPO_4^=$)† (MEQ. PER L.)		PROTEIN‡ (MEQ. PER L.)		SODIUM (MEQ. PER L.)		POTASSIUM (MEQ. PER L.)	
	RESTING	STIMULATED	RESTING	STIMULATED	RESTING	STIMULATED	RESTING	STIMULATED	RESTING	STIMULATED	RESTING	STIMULATED
1. D. G.	0.25	0.56	3.48	11.89	21.42	10.08	0.95	0.59	6.50	30.90	19.90	11.80
2. A. D.	0.44	0.56	10.70	14.89	10.24	8.46	1.00	0.77	7.60	19.10	21.30	21.00
3. A. R.	0.15	0.65	4.47	12.90	11.24	7.56	0.82	1.00	3.30	20.90	24.40	22.10
4. C. S.	0.32	0.60	4.78	14.72	14.22	12.14	0.45	0.68	4.80	19.60	22.10	19.20
5. D. G.	0.20	0.47	4.40	11.89	18.36	11.60	0.76	0.47	9.10	41.70	15.60	13.80
6. E. W.	0.30	0.70	6.14	18.96	14.74	12.42	0.55	0.68	6.10	33.90	23.80	20.00
7. H. C.	0.20	0.48	5.47	12.52	10.44	10.98	1.45	0.59	12.20	41.70	14.10	18.50
8. J. H.	0.14	0.50	6.98	15.97	16.38	11.16	0.76	0.34	6.50	36.50	12.30	20.50
9. E. Y.	0.33	0.80	7.28	8.12	13.14	6.66	1.09	0.68	7.60	30.40	24.60	20.80
10. M. B.	0.30	1.50	4.54	18.49	22.14	14.58	1.02	1.26	10.00	42.60	21.50	25.10
11. C. R.	0.22	0.56	5.88	18.75	10.80	14.40	0.82	0.59	10.00	30.00	18.70	20.50
12. H. P.	0.30	0.71	7.64	18.02	14.94	11.52	1.36	1.00	9.10	25.20	22.60	19.70
13. P. P.	0.33	0.70	7.74	18.19	10.44	10.62	1.53	0.94	16.50	40.90	14.50	15.10
14. M. W.	0.23	0.55	4.85	13.03	13.86	7.56	1.15	1.28	5.40	14.20	10.50	16.40
15. G. E.	0.40	0.90	8.15	17.94	10.08	6.12	0.91	1.04	7.60	19.60	18.20	19.50
16. J. E.	0.40	0.65	7.44	18.43	9.54	9.00	1.41	1.26	15.60	34.60	16.90	15.10
17. K. G.	0.34	0.65	6.95	17.74	10.62	7.92	1.15	0.55	13.00	24.20	17.70	22.60
18. F. S.	0.20	0.57	5.03	15.07	8.82	6.12	1.19	0.47	9.10	41.30	14.80	16.40
19. S. S.	0.24	0.51	5.75	12.84	10.80	9.90	0.55	0.81	13.50	39.10	17.20	18.90
20. L. C.	0.34	0.63	9.23	18.76	9.90	8.46	1.06	0.51	16.00	87.00	14.10	15.90
21. M. A.	0.28	0.46	4.54	13.98	12.78	9.18	0.64	0.51	15.70	56.00	15.60	16.40
22. G. A.	0.28	0.75	9.30	17.22	12.42	10.26	0.75	0.51	13.00	42.60	17.90	17.70
23. M. M.	0.15	0.70	9.05	18.50	12.60	8.10	1.19	0.54	23.90	34.80	22.60	20.50
24. F. P.	0.45	0.65	5.85	15.22	17.82	6.48	0.58	0.38	9.80	38.20	20.20	20.50
25. Z. G.	0.30	0.70	5.50	19.47	12.78	7.74	0.38	0.34	4.35	34.80	17.40	19.00

*Calculated as volumes per cent CO_2 × factor 0.423. This factor combines the conversion of total CO_2 from volumes per cent to milliequivalents per liter and the subtraction of CO_2 present as H_2CO_3 according to its solubility coefficient.

†Calculated as mg. phosphorus per 100 ml. divided by atomic weight of phosphorus, the quotient multiplied by 1.8.

‡Calculated as grams protein per 100 ml. multiplied by Van Slyke factor 2.43. (The Van Slyke factor for blood protein is used in this calculation in the absence of more direct evidence of the base equivalence of salivary proteins.)

concentration. These observations substantiate Heidenhain's original findings that the total percentage of inorganic salts in saliva becomes greater as the rate of secretion is increased.[12]

Both sodium and bicarbonates are present in the extracellular component of blood in forms which may pass readily into and be released by the glands contributing to the formation of human saliva. By comparison, phosphates, proteins, and potassium are primarily intracellular constituents, their presence in saliva representing materials stored in the secretory cells which have been converted into a soluble form and subsequently discharged. The greater concentration of sodium and bicarbonate observed in the saliva samples obtained by stimulation may be the result, therefore, of the difference between the secretory and excretory functions of the salivary glands.

SUMMARY

The greater buffer power of stimulated saliva as contrasted with that of resting saliva in the same person is due to the increase in the quantity of sodium and bicarbonate elaborated by the salivary glands in response to stimulation.

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INHIBITORS IN RELATION TO SALIVARY AMYLASE ACTIVITY: QUININE AND URETHANE

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RECENT work has established that for salivary amylase one phase of the process of heat denaturation† is largely reversible upon rapid recooling[1] or application of high hydrostatic pressure (400 atmospheres).[2] With other enzymes undergoing reversible denaturation, catalysis of this process provides a basis for the action of a selected group of inhibitors, including quinine[3, 4] and ethyl urethane.[5, 6] Inhibition of salivary amylase by chemical agents has not been studied hitherto in relation to such a mechanism, and consequently this investigation of the effects of quinine and urethane was undertaken.

METHOD

The powdered preparation of human salivary amylase used in earlier studies[2, 7, 8] was employed for this investigation. Except where indicated, 2.5 ml. digestion mixtures were prepared containing 0.5 ml. of a solution of amylase previously dialyzed essentially salt-free, 1.0 ml. of soluble starch solution (10 mg. per milliliter of distilled water), and 1.0 ml. 0.025 *N* sodium chloride with or without added inhibitor and buffered to pH 7.0 with 0.01 *M* NaH_2PO_4–Na_2HPO_4, as described elsewhere in fuller detail.[7, 8]

Acidification with HCl was required to dissolve the quinine alkaloid. After neutralization with NaOH and addition to the digestion mixture, the final concentration of NaCl was 0.025 *N*. Accordingly, in each experiment involving quinine, the control digestion mixture was made up to contain 0.025 *N* NaCl.

The experiments on the effect of prolonged contact between amylase and urethane in the absence of NaCl generally involved the use of unbuffered solutions, since the presence of even low concentrations of salt reduced the effect of the inhibitor. Urethane did not cause significant change in the pH of distilled water, and unbuffered urethane and control solutions in distilled water, adjusted where necessary to pH 7, could be used.

The pressure apparatus and the procedure involved in its use have been described.[2]

Amylase activity has been expressed in terms of milligrams of reducing sugar formed from the hydrolysis of the starch substrate within a specified length of time after the addition of the enzyme to the substrate salt solution. Reducing sugar was determined colorimetrically using a Photovolt Lumetron colorimeter, according to the method of Myers, Free, and Rosinski.[9]

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†The general term "denaturation," as used here, refers to a process of inactivation involving a high heat of reaction.

RESULTS

The data given in Table I indicate that quinine ($6.0 \times 10^{-4} M$) and ethyl urethane (0.2 and 0.5 M) are without significant effect on salivary amylase during a twelve-minute digestion period at 37° C. and pH 7.0 in the presence of substrate and adequate NaCl.

TABLE I
EFFECT OF INHIBITORS ON SALIVARY AMYLASE ACTIVITY AT 37° C. IN PRESENCE OF SUBSTRATE

INHIBITOR		REDUCING SUGAR*	INHIBITION
NAME	(MOLES/L.)	(MG./ML.)	(PER CENT)
Blank†	–	0.02	
Control†	–	0.82	
Urethane	0.5	0.78	5
Urethane	0.2	0.78	5
Control‡	–	0.82	
Quinine‡	0.0006	0.82	0

*All digestions were carried out for ten minutes with starch substrate adjusted to 4.0 mg. per milliliter; 0.01 N NaCl; pH 7.0.
†Blank, no amylase; control, amylase present.
‡Concentration of NaCl was 0.025 N.

As the temperature is increased above the optimum for amylase activity (40 to 50° C., the exact point being dependent upon the concentration of salts and protective impurities[1, 10, 11]), inhibition of amylase activity by quinine or urethane becomes marked. The data in Table II show that at 55° C., 6.0×10^{-4} M quinine causes 20 per cent inhibition of amylase activity in a digestion period of twelve minutes under the same conditions of salt and substrate concentration employed for reaction at 37 degrees. In the case of quinine, solubilization of the alkaloid required acidification; subsequent neutralization with NaOH resulted in a final NaCl concentration of 0.025 N in the digestion mixture. With urethane the customary optimal NaCl concentration[10] of 0.01 N could be employed. It has been established that sodium chloride stabilizes amylase[1, 11, 8] and it is therefore likely that the inhibitory effect of 6.0×10^{-4} M quinine would be even greater than 20 per cent at 55 degrees if the lower concentration of NaCl could be employed.

TABLE II
EFFECT OF INHIBITORS ON AMYLASE ACTIVITY AT 55° C. IN PRESENCE OF SUBSTRATE

INHIBITOR		REDUCING SUGAR*	INHIBITION
NAME	(MOLES/L.)	(MG./ML.)	(PER CENT)
Blank	–	0.04	
Control	–	0.83	
Urethane	0.8	0.22	73
Urethane	0.02	0.56	32
Urethane	0.002	0.75	10
Urethane	0.0002	0.76	8
Control†	–	0.97	
Quinine	0.0006	0.71	20

*All digestions were carried out for twelve minutes at 55° C. with starch adjusted to 4.0 mg. per milliliter; 0.01 N NaCl; pH 7.0, except as indicated.
†Digestions in the presence of 0.025 N NaCl.

At 55° C., and in the presence of 10^{-2} *N* NaCl at pH 7.0, urethane caused an inhibition of amylase activity, within the twelve-minute digestion period, to an extent dependent on the concentration of inhibitor. Thus, 8×10^{-1} *M* urethane produced a 73 per cent reduction in activity, 2×10^{-4} *N* urethane produced an 8 per cent reduction, and intermediate concentrations of inhibitor caused graded degrees of inhibition (Table II).

The inhibitory effect of 2.0×10^{-1} *M* urethane at high temperature is not reduced by the application of a pressure of 5,500 lbs. per square inch, as shown by the data in Table III. In the case of other enzymes showing reversible denaturation, inhibition by quinine and urethane is largely mediated by this process, and the degree of inhibition, generally, may be significantly reduced by the application of high hydrostatic pressure. Partial reversibility of amylase inactivation due to high temperature alone is evident in the pressure data given in Table III. However, the degree of reactivation by pressure is the same for the control and urethane-inhibited enzyme (Table III). Consequently, the degree of inhibition remains unaffected by compression, and this suggests that a pressure-sensitive inactivation process does not provide a basis for inhibition by quinine or urethane in the case of salivary amylase.

TABLE III
EFFECT OF PRESSURE ON THE ACTION OF URETHANE AT 60° C.

INHIBITOR CONCENTRATION		P = 15 LBS. PER SQUARE INCH		P = 5500 LBS. PER SQUARE INCH		CHANGE BY PRESSURE (PER CENT)
NAME	MOLES/L.	REDUCING SUGAR* (MG./ML.)	INHIBITION (PER CENT)	REDUCING SUGAR (MG./ML.)	INHIBITION (PER CENT)	
Control	–	0.40		0.70		
Urethane	0.2	0.20	50	0.35	50	0

*All digestions were carried out for twelve minutes with starch adjusted to 4.0 mg. per milliliter; 0.01 *N* NaCl; pH 7.0.

The inhibition of amylase by urethane at high temperatures apparently is not reversed by subsequent cooling and dilution. The data in Table IV show that when amylase inhibited by 8×10^{-1} *M* urethane during a twelve-minute digestion period at 55 degrees and a control amylase solution submitted to the same temperature treatment are separately dialyzed against distilled water at 3 degrees for forty-eight hours, the control enzyme solution still shows marked amylolytic activity in a twenty-minute digestion period at 40 degrees, while the urethane-inhibited amylase shows no activity for the same period of digestion at this temperature.

TABLE IV
EFFECT OF DILUTION ON AMYLASE INACTIVATED BY URETHANE AT 55° C.

	55° C., 12 MINUTES REDUCING SUGAR MG./ML.	40° C., 20 MINUTES REDUCING SUGAR MG./ML.
Control	1.05	0.54
Urethane	0.30	0.0
	Before dialysis 48 hours 3° C.	After dialysis

In the absence of both substrate and sodium chloride, urethane produces a marked increase in the rate of spontaneous inactivation of the amylase, even at 37° C., which appears as a urethane inhibition when substrate and NaCl are admitted to the reaction mixture at 37 degrees and digestion is permitted to proceed. This is demonstrated by the data in Table V. It is also evident from Table V, and has been previously established, that the absence of salt promotes the spontaneous inactivation of amylase.[1, 11, 8] However, even when a control dialyzed free of dissolved salts is used as a reference, 0.5 *M* urethane produces a 50 to 77 per cent inhibition of amylase during a twenty-minute period of contact with the enzyme at 37 degrees and pH 7.0, in the absence of salt and substrate.

TABLE V
EFFECT ON SALIVARY AMYLASE OF PROLONGED CONTACT WITH URETHANE IN ABSENCE OF SUBSTRATE AND SODIUM CHLORIDE

AMYLASE SOLUTION TREATMENT*			DIGESTION MIXTURE†		
INHIBITOR		NaCl CONCENTRATION (MOLES/L.)	URETHANE CONCENTRATION (MOLES/L.)	REDUCING SUGAR (MOLES/L.)	INHIBITION (PER CENT)
NAME	(MOLES/L.)				
Control‡	–	0.017	–	0.61	
Control§	–	–	–	0.31	
Urethane§	0.50	–	0.21	0.15	50
Urethane§	0.50	–	0.21	0.07	77

*Kept 20 minutes at 37° C.
†Digestions with starch 3.0 mg./ml., pH 7.0, 0.007 *N* NaCl, 15 minutes, 37° C.
‡Amylase solution adjusted to pH 7.0 with 0.004 *M* phosphate buffer.
§Amylase solutions unbuffered at pH 7.0, adjusted with NaOH or MCl if necessary.

With sodium chloride present but in the absence of substrate during a predigestion period of up to sixty mintes at 22 or 37 degrees and pH 7.0, 0.83 *M* urethane produces an inactivation of only 5 to 12 per cent. Under the same conditions of time of contact and temperature, 1×10^{-3} *M* quinine produces no measurable inhibition. In each case, activity was measured in terms of the con-

TABLE VI
EFFECT ON SALIVARY AMYLASE OF PROLONGED CONTACT WITH INHIBITOR IN ABSENCE OF SUBSTRATE BUT WITH SODIUM CHLORIDE PRESENT

AMYLASE SOLUTION TREATMENT*				DIGESTION MIXTURE†			
INHIBITOR CONCENTRATION		TEMPERATURE	TIME	INHIBITOR CONCENTRATION	NaCl CONCENTRATION	REDUCING SUGAR	INHIBITION
NAME	(MOLES/L.)	(DEGREES C.)	(MINUTES)	(MOLES/L.)	(MOLES/L.)	(MG./ML.)	(PER CENT)
Control	–	21	60	–	0.01	0.40	
Urethane	0.83	21	60	0.50	0.01	0.38	5
Control	–	21	60	–	0.025	0.40	
Quinine	0.001	21	60	0.0006	0.025	0.38	5
Control	–	37	55	–	0.01	0.32	
Urethane	0.83	37	55	0.50	0.01	0.28	12
Control	–	37	55	–	0.025	0.35	
Quinine	0.001	37	55	0.0006	0.025	0.35	0

*Amylase solutions held at pH 7.0 and .017 *N* NaCl, except the quinine solutions which were adjusted to 0.036 *N* NaCl. The amylase solutions were adjusted with 0.004 *M* phosphate buffer.

†Digestion with starch 4 mg./ml., pH 7.0, 15 minutes, temperature same as for treatment of amylase solution.

centration of reducing sugar formed during a fifteen-minute digestion period at 22 or 37° C. immediately following the period during which substrate was absent (Table VI).

DISCUSSION

Smorodintzev and Novikov[12] have reported that quinine accelerates the amylase-starch reaction. This work, however, is open to the objection that no provision was made for maintaining a concentration of halide salts necessary for optimal, or even near-optimal, amylase activity. Quinine was used as a hydrochloride salt. It is most likely that the acceleration observed by these workers upon the addition of quinine hydrochloride to the starch-amylase solution was due to the added chloride.

In more carefully controlled experiments, Ohlsson[13] found that quinine hydrochloride (1.7×10^{-3} *M*) caused no change in pancreatic amylase activity at 38 degrees and pH 6.89.

The data presented here indicate that quinine in a final concentration of 6.0×10^{-4} *M* exerts no measurable action on salivary amylase at 37 degrees and pH 7.0 in the presence of substrate and 0.025 *N* NaCl. A concentration of quinine of 6.0×10^{-4} *M* causes about 50 per cent inhibition of bacterial luciferase under comparable conditions of temperature and pH.[4]

Urethane (ethyl carbamate) also is without significant inhibitory effect on amylase at 37 degrees in the presence of substrate and adequate NaCl. With 0.5 *M* urethane, an inhibition of 5 per cent was frequently found. In the case of yeast invertase, 0.5 *M* urethane caused 67 per cent inhibition at the temperature and pH for optimal enzyme activity.[6]

The presence of substrate and NaCl, singly or together, appreciably stabilizes amylase against spontaneous inactivation. This inactivation proceeds to some extent even at low temperatures (25° C.) and increases markedly in rate as the temperature is raised.[8, 11] In addition, at all temperatures consistent with enzyme activity, some fraction of the amylase is in a reversibly inactivated state.[1, 2] The extent of the reversible inactivation may be gauged by the effect of high hydrostatic pressure in increasing the activity of the enzyme, since pressure has no measurable effect on the amylase-starch reaction per se[2] and appears to act solely by reversing the volume increase of the inactivation. At 10 degrees, for instance, the reversibly inactivated fraction is relatively insignificant; at 25 degrees it has attained measurable proportions, while at 60 degrees a large fraction of the amylase evidently is in a reversibly inactivated state[2] (Table III).

In the case of bacterial luminescence, particularly, quinine and urethane inhibit enzyme activity largely by promoting or catalyzing such a reversible inactivation process.[4, 5] Thus, with that system, inhibition by urethane or quinine appears to some extent at low temperatures and, with any selected inhibitor concentration, increases as the temperature is raised. Moreover, the inhibition is largely reversed by the application of high hydrostatic pressure, although some fraction of the inhibition is generally mediated by an effect on the irreversible inactivation process, and that fraction is not reversible by pressure.[6]

In the case of salivary amylase, the inhibitory effect of each of these agents is most readily interpreted in terms of an action limited to the spontaneous, apparently irreversible inactivation of the enzyme which catalyzes this process. The evidence for this derives from four main effects. First, in the presence of NaCl and substrate, the inhibition occurs rapidly to any extent only at temperatures where the spontaneous inactivation is prominent and not at temperatures were only native or reversibly denatured amylase predominate. Second, the inhibition caused by these compounds is not retarded by pressure. Third, the inhibition, by urethane at least, is not reversible by dilution. Fourth, when quinine or urethane is permitted to stand in contact with amylase at 37 degrees and pH 7.0 for twenty minutes under conditions which promote the spontaneous inactivation of the enzyme, e.g., in the absence of substrate and protective NaCl, a marked inhibition of activity is noted when substrate and NaCl are admitted. This inhibition, which is apparently due to an acceleration of spontaneous inactivation, is similar to that reported for salivary amylase by urea[7] and appears even at temperatures below 40° C. Contact between drug- and substrate-free amylase in the presence of NaCl at 21 or 37° C. for twenty to fifty-five minutes gives no, or only slight, inhibition when the rate of starch hydrolysis is measured after the period of contact.

In the case of certain enzymes, such as bacterial luciferase and yeast invertase, the reversible inactivation or denaturation process may provide a basis for the action of selected protein-denaturant drugs. The data presented here, however, indicate that with the enzyme, salivary amylase, this reversible denaturation reaction is not of significance as a locus for drug action.

SUMMARY

In neutral solution, quinine and urethane exert a significant effect on salivary amylase only under conditions of temperature and salt concentration which themselves induce amylase inactivation of an apparently irreversible type. The data suggest that the reversible denaturation reaction, which mediates the inhibitory effects of these drugs on a number of other enzyme systems, does not serve as a locus for the inhibitory action of quinine and urethane on amylase. In the case of salivary amylase, the effects of quinine and urethane may be attributed to a catalysis of the spontaneous, apparently irreversible inactivation.

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LEUKOCYTES IN THE SALIVA OF CARIES-FREE AND CARIES-ACTIVE SUBJECTS

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INTRODUCTION

IN SPITE of the fact that phagocytosis of bacteria by certain leukocytes is a property of these cells which has been recognized for many years, the study of the cellular elements of saliva can be considered as a relatively neglected subject in dental literature.

A fairly comprehensive review of the early contributions was presented by Orban and Weinmann.[1] Isaacs and Danielian[2] came to the conclusion that saliva, as it appears at the opening of the ducts of the salivary glands, is practically free of leukocytes and that these cells wander through the oral mucous membrane into the saliva. They found that the number of leukocytes varies between 5 and 150 per cubic millimeter during the course of the day and variations accompany the changes in the blood leukocyte count, the relation being most commonly a reciprocal one, i.e., if the leukocyte count in the blood is high, it is low in the saliva, and vice versa. These authors were unable to find any definite relationship between the epithelial cells in saliva and the count of salivary leukocytes. Stephens and Jones,[3] on the other hand, observed no correlation between the total number of leukocytes in the peripheral blood and in the saliva. Comroe[4] described the cellular elements of saliva in relation to systemic disease; he found no definite correlation, and considered that it was doubtful whether any useful diagnostic information could be gained by examination of specimens of saliva.

Two suggestions have been made as to a possible significance of leukocytes in saliva. First, some authors maintain that by process of disintegration they may release proteolytic enzymes into the saliva, but it is never made clear quite what is the effect of these enzymes. The second suggestion is that they act as phagocytes. Appleton[5] stated that polymorphonuclear leukocytes are constantly passing through the epithelium of the oral mucosa to the free surface. He suggested that these cells are potentially phagocytic and possibly contribute, in part, to the diminution of the bacterial flora of the mouth, a view also held by Bibby, Hine, and Clough.[6] The results of the study by Hammond and Weinmann[7, 8] are interesting in this respect. They found that the average opsonic index in caries-free saliva from eighteen subjects was ten times that of ten subjects who had rampant caries.

Dietz[9] demonstrated the chemotactic influence of human saliva from three subjects upon leukocytes, but did not attempt to correlate intensity of influence with presence or absence of caries.

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Orban and Weinmann[1] dealt principally with the examination of smears of saliva specimens from caries-active and caries-free patients. They found that in the saliva of caries-free persons, there were relatively more epithelial cells and fewer leukocytes than in the saliva of those who were caries-active, and the epithelial cells present in the former were covered by more bacteria than was the case with the latter.

The implications of this work, in our view, are threefold. First, it implies that leukocytes either are broken down more rapidly in caries-free saliva or fail to pass through the mucous membrane in such large numbers, second, that epithelial cells in some way attract bacteria in caries-free mouths to a greater extent than in caries-active mouth, and third, that phagocytosis in caries-free mouths by leukocytes takes place to a relatively small extent. It is true that the larger number of leukocytes in the saliva of caries-active subjects might be explained by a positive chemotaxis of the saliva or organisms on the leukocytes. In the main, however, the evidence of Orban and Weinmann does not favor the suggestion that leukocytes are actively phagocytic in the mouth, and again it is difficult to see how Weinmann can explain the observations on opsonic indices (Hammond and Weinmann[7, 8]) in the light of the results of these experiments. It seems that no report has been published which assists in elucidating these inconsistencies.

Van Kesteren, Bibby and Berry[10] performed experiments to determine whether there was any correlation between the bacterial inhibitory activity of saliva, against standard organisms measured by the "well" technic and any of its constituents. They list a number of materials which were investigated, including leukocytes; no correlation was found between the potency of saliva in the twenty specimens studied and the leukocyte count. No indication was given of the number of subjects who provided the saliva, or of their state of dental caries.

Cattoni[11] studied the distribution of lymphocytes in the epithelium of healthy gingivae and found these cells in between the epithelial cells, particularly in the basal region; he suggests that they may have a "defense" action in inflammatory conditions.

A number of problems suggest themselves as requiring attention in this field, but it was thought that the most urgent was an extension of the observations of Orban and Weinmann on the leukocyte population of salivas from caries-active and caries-free mouths.

METHODS

Saliva specimens were collected from caries-free and caries-active subjects, in a manner which differed in the various experiments to be described. Complete absence of cavities and fillings on careful mirror and probe examination was the criterion by which the former group was chosen and the caries-active subjects all had a number of fillings and at least one open carious lesion.

Where necessary, samples were diluted with one volume of normal saline (0.9 per cent). All specimens were mixed thoroughly by a simple mechanical stirring device. One drop of thoroughly mixed saliva (or saliva plus normal

saline) then was placed as quickly as possible, in order to minimize errors which might arise through sedimentation, on the counting chamber of a hemocytometer slide and the cover glass was lowered. The counting chamber then was examined under a phase contrast microscope.

It became obvious early in this research that, unless criteria were adhered to very carefully, the results could not be reproduced easily because of the fact that, in some salivas, so many cells were in various stages of disintegration. Indeed, some of them were in a condition which made recognition a matter of doubt. This difficulty was often aggravated in salivas with a high bacterial content by the presence of clumps of bacteria which might or might not have been obscuring the presence of a cell.

It was decided, therefore, to adopt the following criteria and count two types of cell: (1) Cells which were intact, whole, and in no way damaged; they might or might not have bacteria, either inside or on the outside. (2) Cells which still could be recognized as such by the presence of a nucleus and at least part of the cytoplasm. Any structure which was the subject of doubt as a leukocyte in our minds was not included at all.

The term "total leukocytes" was applied to the sum of the cells included under Types 1 and 2, and that of "intact leukocytes" to that under Type 1. It was observed that cells in both these categories differed morphologically from the leukocytes seen in fixed and stained blood smears. This difference is the subject of discussion at a later stage of this paper.

The method of counting adopted was designed to ensure the maximum coverage of the counting chamber of the hemocytometer slide. Thirty-seven of the large squares (each equivalent to 1/25 of a square millimeter) were examined in a large Z pattern covering the largest field possible with the slide. By a simple calculation the number of cells per cubic millimeter was obtained.

EXPERIMENTAL

Preliminary Experiments.—A number of preliminary experiments were performed. The first series was designed to investigate the extent to which the counting technic was reproducible. Two samples of saliva were examined. Each specimen was collected over a timed period of five minutes, the subject chewing paraffin wax to stimulate flow; the sample then was thoroughly mixed by the mechanical stirrer and a drop was withdrawn for total leukocyte count. On completion of the count the specimen was stirred again, and a further drop was withdrawn for a second count. The process was repeated eight times with one sample, and six times with another. The number of cells per cubic millimeter was calculated, and the mean of the two samples, their standard deviations, and standard errors of mean were obtained. Table I lists these results. These figures provided the datum line, i.e., the statistical limits of reproducibility of the technic, from which the significance of results to be described could be considered. As compared with variations in the experiments which follow from sample to sample, subject to subject, and group to group, the measure of internal agreement of these experiments on the two samples of saliva will be found to be very satisfactory.

TABLE I

RESULTS OF A NUMBER OF COUNTS OF LEUKOCYTES ON THE SAME SAMPLE OF SALIVA, TOGETHER WITH A STATISTICAL ANALYSIS

	SAMPLE 1 (8 COUNTS) CELLS PER C.MM.	SAMPLE 2 (6 COUNTS) CELLS PER C.MM.
Range	515 to 658	285 to 405
Mean	575	346
Standard deviation	41.99	45.22
Standard error of mean	14.83	18.46

Orban and Weinmann[1] used for their experiments a "loopful" of saliva to obtain the smears with which they worked. The second series of preliminary experiments involved an effort to repeat such a method of collection, by using a sterile platinum wire loop to obtain a sample of saliva from a buccal fold of a number of subjects. It soon was found that specimens obtained in this way were unsuitable for the hemocytometer technic. Apart from the samples being very small and hardly adequate in some cases to permit transference of a drop large enough to cover the field of the instrument, the specimens were loaded very heavily with squamous epithelial cells, presumably removed partly by mechanical action from the buccal fold. The presence of these cells in such large number made accurate counting of leukocytes impossible. It was decided also that any effort to dilute the specimens quantitatively, in order to facilitate counting, would be subject to considerable error. In consequence of these facts, this method of collection had to be abandoned.

In a third series of preliminary experiments, specimens of saliva were obtained from four individuals who were caries-free and two who were caries-active. These samples were collected in the following way: Subjects were asked, without any previous warning, to empty the saliva present in their mouths as quickly as they were able into a specimen tube. The time lag between the request and the collection was minimal and the sample could be said to be as fair a representation in composition of the contents of the mouth at the particular moment of expectoration as was possible.

This collection, which will be referred to as the spontaneous sample, was followed immediately by a wax-stimulated collection, the period being timed to five minutes as accurately as was possible. At the end of the collection the volume produced by the subject was noted. This sample will be referred to as the five-minute sample.

Both of these samples, after dilution with an equal volume of normal saline, were examined in the hemocytometer chamber under a phase contrast microscope. Counts were made of the intact leukocytes and of the total leukocytes.

RESULTS

Table II shows the results of the counts in the spontaneous samples and Table III shows the results of those in the five-minute samples. The first point arising from a study of these tables is that the results are not in agreement with those of Orban and Wienmann[1] who, using smears of saliva specimens,

found fewer leukocytes in caries-free salivas. Tables II and III show that the count of intact leukocytes, in both the spontaneous and five-minute specimens, in saliva from caries-free subjects tended to be higher than that found in the specimens provided by the caries-active group. The same relationship is found to apply to the total leukocytes of the spontaneous samples, but the five-minute samples showed counts which were much alike.

There are a number of criticisms to be applied to the results shown in these tables. First, the number of subjects studied is small, particularly the group of caries-active subjects. We wish, however, to emphasize the preliminary nature of these experiments. Second, variations in counts of specimens taken at different times from the same subject are often large, the deviation from the mean being greater in the case of the spontaneous samples than in the five-minute samples. It is probable that this latter observation arose as the result of the effect of portions of saliva, particularly in the spontaneous samples, accumulated in parts of the mouth normally out of the salivary circulation.

At the same time it was observed that the specimens, again the spontaneous samples especially, often contained a large amount of debris (e.g., food residues, plaque material, precipitated mucin, epithelia, etc.) which tended to obscure the hemocytometer field and decrease the accuracy of counting.

The indications were, then, that it might be preferable in the next series of experiments, which were designed to repeat and extend those described using a larger number of subjects, to start the collection of samples under standard conditions after a cleaning procedure involving rinsing the mouth and brushing the teeth. Such a technic would provide a further advantage in that the validity of any difference between the leukocyte populations of salivas from caries-free and caries-active groups of subjects which might be observed under these conditions would become more apparent.

Further Studies on the Leukocyte Population of Saliva.—Each subject was provided with a new toothbrush and was instructed to brush the teeth and gingivae thoroughly with repeated rinsing out of the mouth for a period of two minutes. Immediately after the completion of the cleansing operation, the time was noted and the five-minute wax-stimulated collection was commenced.

In this series the specimens were not diluted, but after mechanical stirring were examined directly under a phase contrast microscope. Hemocytometer counts were made, as before, of the intact and total leukocytes. The volumes of samples had been noted. Five specimens were taken at approximately weekly intervals from eleven individuals who were caries-free, sixteen who were caries-active, and two who were caries-low. These latter subjects had had only one or two fillings in their permanent teeth during their lives, none of which had been attended to within six months of taking the specimens; they had been pronounced free from active caries by a clinical examiner.

RESULTS

Tables IV, A and IV, B show the figures obtained for the average of the counts, at the five sittings, of intact and total leukocytes, the average volumes

TABLE II

LEUKOCYTE COUNTS IN SPONTANEOUS SPECIMENS OF SALIVA

SUBJECT NUMBER	STATE OF CARIES	INTACT LEUKOCYTES (CELLS PER C.MM.) COUNTS	AVERAGE	TOTAL LEUKOCYTES (CELLS PER C.MM.) COUNTS	AVERAGE
310	caries-free	216.5, 365.0, 40.6, 0,	155.5	4340.0, 1945.0, 3595.0, 1216.0, 2652.0	2749.6
311	caries-free	13.5, 148.8, 135.0, 94.6, 148.8	108.1	1054.0, 797.0, 1569.0, 2731.0, 1892.0	1608.6
312	caries-free	395.0, 81.1, 148.8, 13.5, 81.1	143.9	3565.0, 1122.0, 2072.0, 1765.0, 1786.0	2062.0
313	caries-free	243.0, 108.2, 230.0, 189.0, 0	154.0	5820.0, 3895.0, 5300.0, 3895.0, 2742.0	4330.4
		Average	140.4	Average	2687.7
68	caries-active	351.5, 175.8, 40.6, 27.0, 0	119.0	2081.0, 2822.0, 1110.0, 838.0, 1798.0	1729.8
324	caries-active	27.0, 41.7, 67.6, 27.0, 40.6	40.8	833.0, 875.0, 1216.0, 243.0, 746.0	782.6
		Average	79.9	Average	1256.2

TABLE III

LEUKOCYTE COUNTS IN FIVE-MINUTE SPECIMENS OF SALIVA

SUBJECT NUMBER	STATE OF CARIES	INTACT LEUKOCYTES (CELLS PER C.MM.) COUNTS	AVERAGE	TOTAL LEUKOCYTES (CELLS PER C.MM.) COUNTS	AVERAGE
310	caries-free	78.0, 27.0, 67.5, 177.5	87.5	485.0, 390.5, 392.0, 256.5, 564.0	417.6
311	caries-free	108.0, 257.0, 83.5, 97.0, 181.0	145.3	892.0, 622.0, 597.5, 417.0, 569.0	619.5
312	caries-free	121.5, 55.5, 83.5, 13.5, 94.5	73.7	419.0, 431.0, 264.0, 122.0, 473.0	341.8
313	caries-free	121.5, 135.0, 67.5, 176.0, 230.0	146.0	811.0, 1690.0, 703.0, 1447.0, 892.0	1108.6
		Average	113.1	Average	621.9
68	caries-active	135.0, 108.0, 0, 0, 13.5	51.3	1311.0, 419.0, 459.0, 594.0, 311.0	618.8
324	caries-active	13.5, 55.5, 143.0, 27.0, 54.0	58.6	703.0, 750.0, 1358.0, 311.0, 473.0	719.0
		Average	54.9	Average	668.9

produced, and the PMA indices of the subjects, to be referred to shortly. The averages and ranges also are shown in these tables, a consideration of which leads to the following conclusions:

TABLE IV, A

RESULTS OF EXPERIMENTS IN WHICH THE MOUTH WAS CLEANSED BEFORE COLLECTION OF SALIVA SAMPLES FROM CARIES-FREE AND CARIES-LOW SUBJECTS

SUBJECT NUMBER	AVERAGE OF INTACT LEUKOCYTES (CELLS PER C.MM.)	AVERAGE OF TOTAL LEUKOCYTES (CELLS PER C.MM.)	AVERAGE VOLUME PRODUCED IN 5 MINUTES (MILLILITERS)	PMA INDEX
CARIES-FREE				
310	69.4	202.2	19.6	0-0-0
311	45.8	152.9	13.8	0-0-0
312	67.9	173.7	15.6	0-0-0
313	216.3	1210.8	7.5	2-1-0
314	101.3	349.8	18.9	5-4-0
315	44.5	384.6	16.3	10-9-0
316	63.7	679.1	9.2	0-0-0
317	200.0	804.0	7.0	1-0-0
318	312.0	782.0	5.5	4-0-0
319	10.8	109.5	7.0	0-0-0
95	105.4	543.0	8.0	8-4-0
Average	112.5	490.1	11.7	
Range	10.8-312.0	109.5-1210.8	5.5-19.6	
CARIES-LOW				
322	54.9	265.3	11.6	5-2-0
323	102.7	779.6	12.7	4-2-0
Average	78.8	522.5	12.2	
Range	54.9-102.7	265.3-779.6	11.6-12.7	

1. The average intact leukocyte count in the caries-free group was nearly four times that observed for the caries-active group, with the caries-low group (far too few in number for results to be of any great significance) in between.

2. The ranges of the intact leukocyte counts of the caries-free and the caries-active groups did overlap, but statistical analysis of the individual counts (not the averages shown in the tables) demonstrated that the difference of the means was highly significant ($t = 6.24$); for significance at $n = 0.001$, $t = 3.373$ (Fisher and Yates[12]).

3. The averages of the total leukocytes were close, but the caries-active mouths showed a few less. The ranges were very wide, and statistical analysis showed that the difference of the means was not significant.

4. In the caries-free salivas about 23 per cent of the cells present belonged to the intact class, whereas in the caries-active group the figure was approximately 7 per cent.

5. The volume secreted by the subjects is not related to the larger number of intact cells observed in the caries-free group. While the latter showed a figure four times as great as that of the caries-active group, the volume ratio was 1.7 to 1. Thus, in spite of the larger volume of saliva produced, the count of intact cells per cubic millimeter was greater in the caries-free specimens.

TABLE IV, B

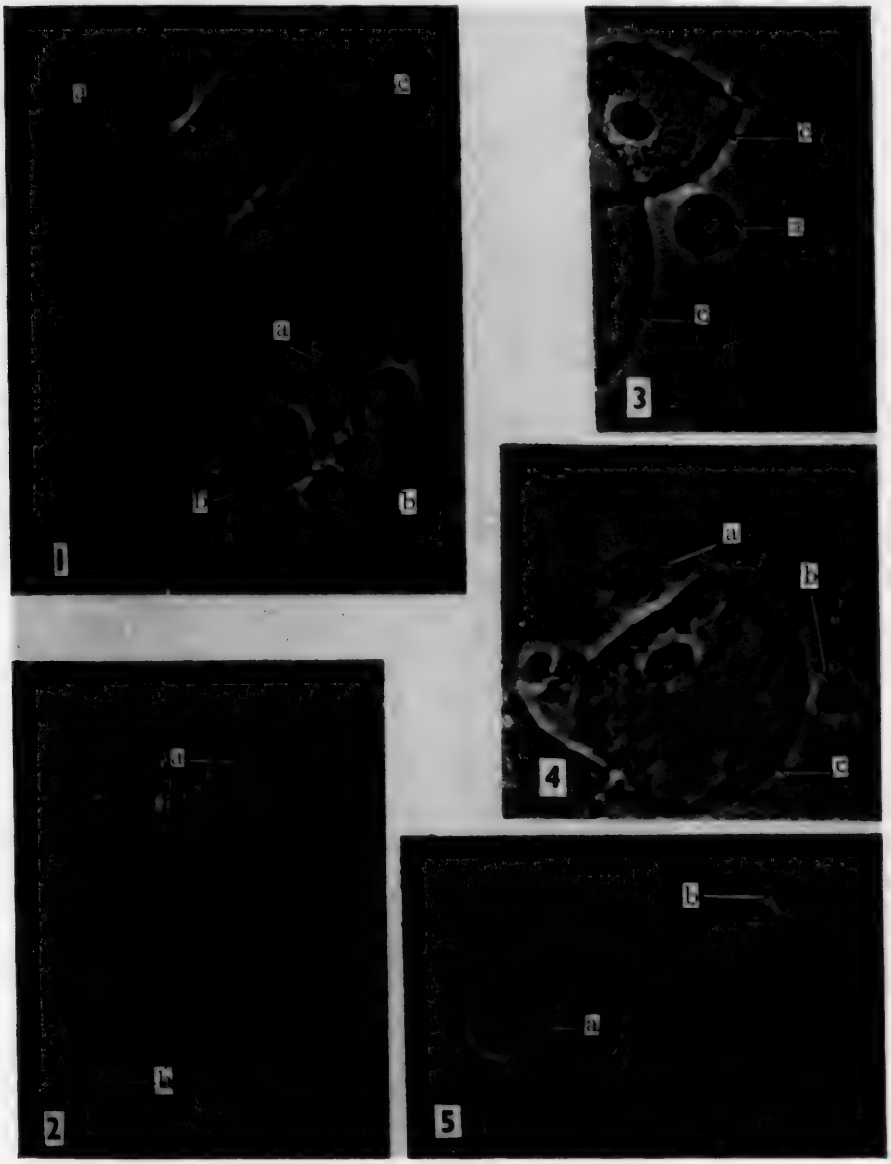
RESULTS OF EXPERIMENTS IN WHICH THE MOUTH WAS CLEANSED BEFORE COLLECTION OF SALIVA SAMPLES FROM CARIES-ACTIVE SUBJECTS

SUBJECT NUMBER	AVERAGE OF INTACT LEUKOCYTES (CELLS PER C.MM.)	AVERAGE OF TOTAL LEUKOCYTES (CELLS PER C.MM.)	AVERAGE VOLUME PRODUCED IN 5 MINUTES (MILLILITERS)	PMA INDEX
68	15.1	565.2	6.2	1-0-0
324	22.3	301.4	6.7	2-0-0
97	10.9	832.2	12.6	3-0-0
320	5.4	129.7	12.0	3-0-0
321	10.9	172.4	5.2	0-0-0
325	12.2	327.1	5.6	5-4-0
326	29.7	516.5	2.0	0-0-0
327	15.0	236.6	9.3	6-3-0
328	23.0	354.0	6.1	2-2-0
329	89.0	415.0	8.7	5-5-0
330	25.6	247.4	5.3	0-0-0-
331	74.3	662.4	8.2	0-0-0-
332	43.2	502.5	3.1	4-0-0
333	21.6	229.8	6.3	5-2-0
98	52.7	1128.0	6.2	5-4-2
67	51.5	467.3	6.4	1-1-0
Average	31.4	443.0	6.9	
Range	5.4-89.0	129.7-1128.0	2.0-12.6	

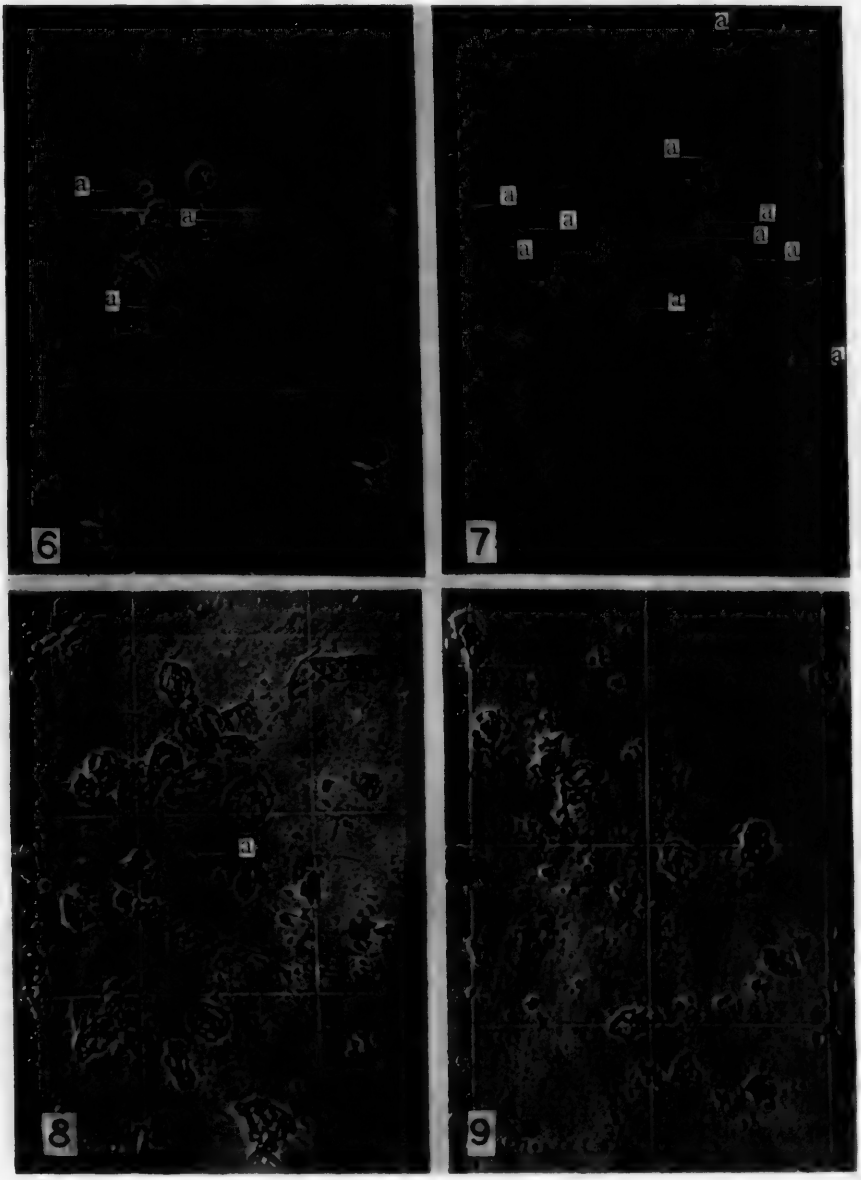
Photomicrographs were prepared of at least one hemocytometer preparation of saliva from each subject, and a selection of these is presented. Figs. 1 to 5 illustrate intact leukocytes and leukocytes in varying states of disintegration, and Figs. 6 to 9 show typical low-power fields of preparations of saliva from caries-free and caries-active subjects. A striking difference in general constituents will be seen, but we wish to draw attention particularly to the feature which is the main subject of this paper, the count of intact leukocytes.

The State of the Gingivae of the Subjects.—It seemed possible that the condition of the gingivae might be related to the cell population of the saliva, since inflammation of the supporting tissues would lead to the presence within them of large numbers of leukocytes, some of which might escape into the mouth. It was clearly desirable to determine whether the number of intact leukocytes in the saliva was related to the condition of the subject's gingivae, particularly as there is a widely held view that those who are caries-free are more prone to inflammatory conditions of the gingivae.

As a check on this point, our subjects were examined and records were made of their periodontal condition in accordance with the technic of Massler, Schour and Chopra.[13] The results of this examination in terms of the PMA (papilla, marginal, attachment) index are seen in the last column of Tables IV, A and IV, B. Comparison of the numbers of intact leukocytes in the saliva of each subject with his PMA index shows that there is no consistent correlation. It is possible, however, that in some cases (e.g., Subjects 329 and 98, who, although caries-active, had high intact cell counts and high PMA indices), failure to conform to the observed relationship of caries to intact cell population could be explained by the condition of the gingivae.



Figs. 1 to 5.—Photomicrographs of hemocytometer preparations of saliva. Phase Contrast ×525, reduced ⅛. *a*, Intact leukocytes; *b*, leukocytes in varying states of disintegration; *c*, epithelial cells.



Figs. 6 to 9.—Photomicrographs of hemocytometer preparations of saliva from caries-free subjects, 6 and 7, and from caries-active subjects, 8 and 9. Phase Contrast ×125, reduced ⅛. a, Intact leukocytes. A considerable difference is to be observed between the pictures presented by the two groups in both intact leukocyte count and other elements.

It would seem, therefore, that the differences observed in the numbers of intact leukocytes in the saliva of the groups studied cannot be related in general to the PMA factor, although it is likely that in individual cases the periodontal condition may override the caries-intact cell relationship.

DISCUSSION

Most other workers in this field have tacitly assumed that the cells they observed, which are similar to those we have described, were leukocytes, despite the fact that they did not conform morphologically to the leukocytes to be seen in fixed and stained blood smears. This is illustrated clearly in Figs. 1 to 5. The difference affects both the nucleus and the cytoplasm. The former does not present the usual structure seen in a polymorphonuclear leukocyte, the lobes are more often than not fused together, and granules are to be seen in a constant state of motion (the preparation, of course, is vital). Lymphocytes have a larger volume of cytoplasm than those seen in blood smears, the nucleus is round and the cytoplasm appears agranular, and it would be difficult to express a confident opinion distinguishing these cells from the counterparts of large lymphocytes and monocytes in saliva. We have not been able to distinguish with certainty eosinophils or basophils from neutrophils in hemocytometer preparations of saliva under the phase contrast microscope.

The above observations apparently are not confined to the study of vital preparations, for the photomicrographs presented by Orban and Weinmann[1] also indicate a change in morphology in the case of fixed and stained smears of saliva, an observation which we have been able to confirm.

While it is always possible that these cells are not leukocytes at all, it is difficult to envisage what other type of cell they might be. The alteration from the usual picture presented in blood may be the result of a number of factors. Cattoni[11] described an alteration in morphology of lymphocytes in their passage through the epithelium; Laquer[14] reported that leukocytes become swollen and their nuclei become fused, losing their lobulated appearance in the slightly hypotonic medium of his saliva specimens; Isaacs and Danielian[2] describe phases through which leukocytes go in the process of losing their vitality, which are not unlike those we have observed.

The main conclusions we have drawn from our studies are that the percentage of intact salivary leukocytes is significantly greater in caries-free than in caries-active saliva, and that the total number of leukocytes is similar in both types of saliva.

It is not yet known whether this higher percentage of cells which are intact in caries-free saliva is caused by increased numbers of cells entering saliva or by a slower rate of disintegration. Experiments which it is hoped will make it possible to decide between these alternatives are now in progress.

Another point which remains undecided is whether the greater proportion of intact cells in caries-free saliva is the result or the cause of the reduced

bacterial activity in the caries-free mouth. This question may be answered perhaps when more information has been gained about the factors controlling the entry of leukocytes into, or their destruction by, saliva.

It is of interest to note that a statistical analysis of the secretion rates of our subjects shows that the difference between the means observed in the caries-free and caries-active groups was highly significant. This is in keeping with the results of Trimble, Etherington, and Losch[15] who found by studying 107 patients that those whose secretion rate was less than average developed nearly twice as many smooth surface cavities over a period of twelve months as those with a secretion rate above average. Hewat[16] also made similar observations on caries-free and caries-active groups of children.

SUMMARY

1. The technic of phase contrast microscopy has been applied to the examination of samples of saliva on a hemocytometer slide.

2. Leukocyte counts of samples of saliva from caries-free, caries-active, and caries-low subjects were obtained.

3. As the majority of these cells in saliva were present in varying stages of disintegration, it was decided to divide them into two groups: (1) cells which were intact and in no way damaged, and (2) the total count of all cells which were in a recognizable state, including intact cells.

4. The count of intact cells in the group of caries-free subjects was approximately four times greater than that from the caries-active group.

5. The count of total cells showed that no statistically significant difference was to be observed between the groups.

6. The volumes of saliva secreted by our caries-free subjects were significantly greater than those produced by the caries-active group.

We wish to express our thanks to Mr. F. E. Hopper of the Newcastle upon Tyne Dental Hospital for very kindly conducting the periodontal investigations described in this paper. We are also glad to record our appreciation of the help given by Professors R. W. Lovell and G. E. M. Hallett for putting us in touch with some of the caries-free subjects and for conducting mirror and probe examinations on others. Finally, our thanks are due the subjects themselves who showed such keenness and cooperation in providing the samples of saliva.

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GERMICIDAL ACTION OF CETYL PYRIDINIUM CHLORIDE ON BETA HEMOLYTIC STREPTOCOCCI

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THE literature on the quaternary ammonium germicides has grown rapidly in the last few years and was reviewed completely in two recent books.[1, 2] However, one of the important characteristics of this group of compounds which has not received adequate recognition is their remarkably rapid germicidal action. Thompson, Isaacs, and Khorazo[3] showed that Zephiran killed the test organisms in fifteen seconds. Mallman, Kivela, and Tumer[4] found that Roccal diluted 1:10,000 killed *Staphylococcus aureus, Escherichia coli,* and *Micrococcus caseolyticus* in five seconds. In 1948 Gardner[5] employed alcoholic solutions of Zephiran and Cetrimide (cetyl trimethyl ammonium bromide) for topical application and observed that they virtually disinfected the skin within thirty seconds. In the same year Johns[6] found that alkyldimethylbenzyl ammonium chloride showed a 99 per cent killing of *Staphylococcus aureus* within fifteen seconds in the presence of 10 per cent skimmed milk.

There are many applications for germicides where extremely rapid action is essential if satisfactory results are to be obtained. For example, the infected surfaces of the body which are continuously bathed with secretions require rapid disinfection in order to obtain adequate benefit from the germicide before it is washed away or diluted beyond its effective concentration. Thus, the infected oral mucous membrane is best treated with a germicide which kills the bacterial invaders as rapidly as possible. In order to determine how rapidly cetyl pyridinium chloride kills bacteria which are very frequently the cause of throat infections, a study was made of the speed of its action on beta hemolytic streptococci. For this purpose thirty-one strains of beta hemolytic streptococci, freshly isolated from throat cultures obtained in three widely separated areas in this country, were obtained.* The well-known C203 strain of hemolytic streptococci also was included for comparison.

In order to determine whether or not other substances commonly used in oral antiseptics, e.g., flavor oils, alcohol, dyes, etc., interfered with the rapid germicidal action of cetyl pyridinium chloride, an oral antiseptic containing 0.025 per cent cetyl pyridinium chloride, 15 per cent alcohol, certified dyes, and flavor oils, and buffered to pH 7.6 was included in this study.

EXPERIMENTAL

The test organisms (beta hemolytic streptococci) were incubated twenty-four hours at 37° C. in a beef infusion broth containing 6 per cent of Armour's

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peptone which supported luxurious growth. The cultures showing homogenous turbidity were diluted with broth, when necessary, to a uniform standard containing 38 to 51 million colonies per cubic centimeter, and 0.5 c.c. was added to 5.0 c.c. of the antiseptic solutions maintained at 37° C. in sterile 15 × 150 mm. test tubes. The tubes were agitated vigorously and samples were transferred with a 4 mm. loop after ten and fifteen seconds' contact to tubes of letheen beef infusion broth.[7] These subculture tubes were incubated at 37° C. for seventy-two hours before the presence or absence of visual growth was recorded. Three separate solutions were tested, viz., 0.025 per cent cetyl pyridinium chloride in buffered (pH 7.6) water, 0.025 per cent cetyl pyridinium chloride in buffered (pH 7.6) water containing 15 per cent ethyl alcohol and the oral antiseptic described previously. A sterile water control was also included in each test. The results of this test are shown in Table I.

TABLE I

NUMBER OF STRAINS OF HEMOLYTIC STREPTOCOCCI SHOWING THE PRESENCE OR ABSENCE OF GROWTH AFTER CONTACT WITH ANTISEPTIC SOLUTIONS OR WATER CONTROL

TEST SOLUTIONS	CONTACT TIME			
	10 SECONDS		15 SECONDS	
	GROWTH	NO GROWTH	GROWTH	NO GROWTH
0.025% C.P.C.* (water)	9	23	2	30
0.025% C.P.C.* (alcohol-water)	3	29	0	32
Oral antiseptic	2	30	0	32
Water (control)	32	0	32	0

*C.P.C.—Cetyl pyridinium chloride.

These results indicate that the oral antiseptic kills the organisms in all of the streptococcus strains used after fifteen seconds' contact, and thirty of the thirty-two strains after ten seconds' contact. It is interesting to note that the addition of alcohol to the cetyl pyridinium chloride appears to enhance its speed of action. Control tests with 15 per cent alcohol in water also were run, and growth was found in all subculture tubes after fifteen seconds' contact.

It has long been recognized that the germicidal action of the quaternary ammonium compounds, like that of most other germicides, is reduced in the presence of organic matter. These compounds have been tested with added serum, milk, and ascitic fluid,[1] but no published reports have been found indicating the effect of saliva on their germicidal action. Since the efficacy of an oral antiseptic may be influenced by the action of saliva upon it, the following study was made to determine the effect of saliva on cetyl pyridinium chloride. Saliva, pooled from four subjects, was sterilized by Seitz filtration and 0.5 c.c. added to 5.0 c.c. of the undiluted antiseptic solutions. Five-tenths of a cubic centimeter of each of the thirty-two streptococcus strains was used as the inoculum, and the remainder of the test was carried out as described previously. A total of 331 individual tests were run in which saliva was added to tubes with 0.025 per cent cetyl pyridinium chloride in water, 0.025 per cent cetyl pyridinium chloride in 15 per cent alcohol or the oral antiseptic. In 270 (82 per cent) of the tests the addition of saliva did not affect the

speed of the germicidal action of the antiseptic. in 26 (8 per cent) of the tests the killing time was reduced, and in 35 (10 per cent) of the tests the killing time was increased. It would seem likely that in most cases the presence of 10 per cent saliva had no effect on the speed of the germicidal action of the cetyl pyridinium chloride. No satisfactory explanation is evident for the tests where the antiseptic action appeared to be enhanced or decreased, other than that it may be the result of "skips" and "wild pluses" commonly observed when testing quaternary antiseptics by this method.[8, 9] In an unpublished study, Carter, Foter, and Nisonger[10] suspended the test organisms in saliva prior to inoculation into the cetyl pyridinium chloride solution and found that the saliva consistently enhanced the activity of the cetyl pyridinium chloride.

DISCUSSION

There has been a considerable amount of disagreement among laboratory workers concerning the proper in vitro method for testing the germicidal action of the quaternary ammonium compounds.[1] The method employed in this study is a modification of the procedure outlined by Ruehle and Brewer[11] wherein the test organisms, the contact time intervals, and the subculture medium have been adapted to the requirements of this investigation. The use of letheen broth to inactivate any possible carry-over of the cetyl pyridinium chloride and the replication of the experiment by using thirty-two different strains of the test organisms provide considerable confidence in the results obtained. It is of interest to note that the C203 streptococcus strain, which has been employed extensively in chemotherapeutic tests, was killed in ten seconds like most of the other strains.

SUMMARY

Thirty-one strains of beta hemolytic streptococci, freshly isolated from throat cultures, and a C203 strain were killed after 10 to 15 seconds' contact with 0.025 per cent cetyl pyridinium chloride in water, with 0.025 per cent cetyl pyridinum chloride in 15 per cent alcohol, or in a mouthwash using a modified phenol coefficient type of test. When 10 per cent sterile saliva was added to the antiseptic solutions, it did not affect the speed of germicidal action in 82 per cent of the tests, while in the remainder of the tests activity was increased or decreased. The results of this study suggest that a rapidly acting, nontoxic germicide, such as cetyl pyridinium chloride should be effective in an oral antiseptic solution which may kill the infecting bacteria before it is washed away or diluted beyond its effective concentration.

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THE EFFECT OF SODIUM PARA-AMINO SALICYLATE AS A GROWTH FACTOR FOR LACTOBACILLI

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A CONSIDERATION of the dental health of patients at the Brecksville Veterans Hospital introduced the question of the caries incidence in patients who have been administered sodium para-amino salicylate. This chemical, the sodium salt of para-amino salicylic acid, is used together with streptomycin in the treatment of tuberculosis. It was believed that, because para-amino salicylic acid is essentially para-amino benzoic acid with an additional hydroxyl group, microorganisms of the mouth might utilize the sodium para-amino salicylate as a substitute for para-amino benzoic acid as a growth stimulant. Jordan and Burrows[1] stated that para-amino benzoic acid is not an essential growth factor for *Lactobacillus casei* or *Staphylococcus aureus*. Shankman[2] proposed that the growth factor could become essential if the medium is incomplete.

Lactobacillus casei (formerly *Lactobacillus acidophilus* 4646), isolated from saliva, was chosen as the test organism because of its known association with dental caries. Visual observation indicates that this organism grew as well in the synthetic broth which contained no para-amino benzoic acid, as in this broth when para-amino benzoic acid and sodium para-amino salicylate (.01 mg. per milliliter) were added separately. *Staphylococcus aureus, Escherichia coli,* and two additional strains of lactobacilli also grew in all of these media.

However, plate counts of the *Lactobacillus casei* grown in dextrose broth, to which para-amino benzoic acid or sodium para-amino salicylate were added to a concentration of .01 mg. per milliliter, indicated a greater stimulation by the sodium para-amino salicylate than the para-amino benzoic acid.

In subsequent experiments sodium para-amino salicylate was added in various dilutions. Both dextrose and a synthetic broth containing 2, 4, 6, and 10 mg. per milliliter of sodium para-amino salicylate were made. The concentrations were made from a stock solution containing 20 mg. per milliliter of sodium para-amino salicylate and the volumes were equalized by the addition of a .85 per cent saline solution. No stimulation was found when the concentrations of sodium para-amino salicylate were in the range of 2, 4, and 6 mg. per milliliter, whereas at 10 mg. per milliliter inhibition was shown.

With this same range of 2 to 10 mg. per milliliter of sodium para-amino salicylate, no stimulation was visible when the solutions were allowed to diffuse through porcelain cylinders placed on the seeded tomato juice agar plate (Oxford cup method).

The effect on glycolysis was observed in the Warburg respirometer, using saliva as the source of microorganisms and glucose as an added substrate.

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When sodium para-amino salicylate was added to make concentrations of .125 mg. per milliliter, a slight inhibition of activity was noted but after a period of forty minutes no significant difference was seen.

SUMMARY

In pure culture the addition of 2.4 and 6 mg. per milliliter of sodium para-amino salicylate to a dextrose or synthetic broth (without para-amino benzoic acid) gave no stimulation to the growth of *Lactobacillus casei*, while at higher concentrations inhibition was visible. With similar concentrations neither stimulation nor inhibition was demonstrable with the Oxford cup method. A concentration of .125 mg. per milliliter did not alter the rate of glycolysis as measured by the Warburg respirometer.

The availability of para-amino benzoic acid in saliva might minimize the possibility of utilization of sodium para-amino salicylate by mouth organisms.

These results would indicate that sodium para-amino salicylate is not an important factor in the stimulation of growth of this organism or in the rate of glycolysis believed associated with dental caries.

The authors express appreciation for the use of the laboratories of Western Reserve University and to Dr. T. J. Hill, Professor of Oral Pathology, Western Reserve University and Veterans Administration consultant in Oral Pathology, for his counsel.

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THE ESTIMATION OF SAMPLE SIZE IN EXPERIMENTS

I. Using Comparisons of Averages

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ONE of the problems confronting an investigator who employs inductive reasoning is how large a sample is necessary to test a particular hypothesis adequately. This is more than a theoretical problem, since the size of the sample to be used will determine, to some extent, the amount of funds requested in a grant, the type of experimental animal to be utilized, and, in some cases, even the type of experiment to be performed.

Many investigators have recognized the need for consultation with someone who may be able to assist them in determining how many cases or animals or patients (i.e., size of sample) are necessary in their contemplated experiment. The investigator will often phrase the question as, "How many cases do I need to make my experiment mean something?" This question has no answer. A more careful phrasing, such as, "How many cases do I need to have a significant result?" still has no answer. What is needed is information as to what contemplated result is considered important, and a certain amount of preliminary data. Such information may have been gained through previous experience with the problem under investigation, either by the individual or others, or as a result of a preliminary or pilot study.

In other words, before a definite answer can be given about the necessary sample size of an experiment, some clue as to how the results are going to run must be available. As a matter of fact, this is the usual sequence in most laboratories: some preliminary studies are performed before the larger, more definitive experiment is set up.

There are a number of different types of experimental designs which can be employed in the dental research laboratory. One of the most frequently employed types is that in which two independent groups of cases are studied and measurements are made on each individual in the two groups. The final comparison is made in terms of the difference between the average measurement of each group. Thus, for example, we can take the problem in which a particular chemical constituent of the saliva is studied in a group of individuals with caries and in a group which is caries-free (Hess and Smith).[1] Needless to say, the two groups chosen should be comparable with respect to all characteristics (i.e., age, sex, general health status, etc.) with the exception of their susceptibility to dental caries. A preliminary study might be performed first and the readings tabulated for each group (Table I).

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TABLE I

ASCORBIC ACID CONTENT OF SALIVA (MG. PER 10,000 C.C.)* OF 9 CARIES-FREE PATIENTS AND 9 CARIES-ACTIVE PATIENTS (HESS AND SMITH)**

CARIES-FREE PATIENTS (X_1)	CARIES-ACTIVE PATIENTS (X_2)
20	19
31	9
14	13
19	20
21	21
17	15
13	12
24	19
22	18
Total $\Sigma x_1 = 181$	$\Sigma x_2 = 146$

*The data in the original paper were expressed in terms of mg. per cent. In order to remove the tediousness of placing decimal points, the dimensions have been changed to mg. per 10,000 c.c. by multiplying the original data by 100.

**These data have been selected for illustrative purposes only, and any conclusions drawn do not necessarily pertain to the results of the orginal study as a whole.

The investigator then computes the average reading for each group as $\bar{x}_1 = \frac{\Sigma x_1}{N_1} = \frac{181}{9} = 20.1$ mg. in the caries-free group, and as $\bar{x}_2 = \frac{\Sigma x_2}{N_2} = \frac{146}{9} = 16.2$ mg. in the caries-active group. The difference between the two means is $20.1 - 16.2 = 3.9$ mg. In order to perform the usual significance test upon the difference between the averages of two independent samples of such small size, the following procedure may be employed.

The squared standard deviation of the readings in each group can be obtained from the formula:

$$s^2 = \frac{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}{N - 1}$$

where Σx^2 is the sum of each individual reading squared, $(\Sigma x)^2$ is the squared total of the individual readings in each group, and N is the number of readings in that group. Thus:

Caries-Free	*Caries-Active*
$\Sigma x_1 = 181$	$\Sigma x_2 = 146$
$\Sigma x_1^2 = 3877$	$\Sigma x_2^2 = 2506$
$N_1 = 9$	$N_2 = 9$
$(s_1)^2 = \frac{3877 - \frac{(181)^2}{9}}{8}$	$(s_2)^2 = \frac{2506 - \frac{(146)^2}{9}}{8}$
$(s_1)^2 = \frac{236.99}{8}$	$(s_2)^2 = \frac{137.56}{8}$

These two squared standard deviations then are averaged, according to the formula:

$$\bar{s}^2 = \frac{(N_1 - 1)(s_1)^2 + (N_2 - 1)(s_2)^2}{(N_1 - 1) + (N_2 - 1)} = \frac{236.99 + 137.56}{8 + 8} = 23.41.$$

The comparison of the two means is made in terms of

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S.E._{\bar{x}_1 - \bar{x}_2}}$$

where the sign of $(\bar{x}_1 - \bar{x}_2)$ is not taken into account here, and $S.E._{\bar{x}_1 - \bar{x}_2}$ is the standard error of the difference between the means and may be obtained as:

$$S.E._{\bar{x}_1 - \bar{x}_2} = \sqrt{\bar{s}^2\left(\frac{1}{N_1} + \frac{1}{N_2}\right)} = \sqrt{23.41\left(\frac{1}{9} + \frac{1}{9}\right)} = 2.3 \text{ mg.}$$

Then, $t = \frac{20.1 - 16.2}{2.3} = 1.70$, at $(N_1 - 1) + (N_2 - 2) = 16$ degrees of freedom. From standard tables[2] of t, we find that at 16 degrees of freedom the values of t exceeded only 10 per cent and 5 per cent of the time by chance alone ($t_{10\%}$ and $t_{5\%}$) are 1.75 and 2.12, respectively. The difference between the average ascorbic acid level in the two groups (3.9 mg.) thus can occur more than 10 per cent of the time through the action of chance factors alone, and it is termed "statistically not significant." The null hypothesis being tested, that there is no difference between the average salivary ascorbic acid level of caries-free and caries-active individuals, therefore cannot be rejected.

The investigator may examine his results and may feel that, while the difference in average salivary ascorbic acid is not significant, it is suggestive. Further, he may feel that if he were to expand his experiment to include more cases in each group, the difference in salivary ascorbic acid would become statistically significant. The question to be answered, then, is how many patients are needed.

This question can be answered since we have some preliminary information about the way in which the results occur. What is first needed, however, is a clear statement as to what difference in average salivary ascorbic acid is to be statistically significant. The investigator must realize that the average difference of 3.9 mg. will vary as more patients are added to the two groups. From the preliminary study it is known that the variation of this difference in means ($S.E._{\bar{x}_1 - \bar{x}_2}$) is 2.3 mg. Therefore, we can set up confidence limits around the average difference (3.9 mg.) within whose borders the true difference in means will be contained a certain percentage of the time. From the standard tables of t, it already has been found that at 16 degrees of freedom a value of $t = 2.12$ will be exceeded 5 per cent of the time through the action of chance factors alone. Looking at this another way, the value of t will be less than 2.12 (either + or −) 95 per cent of the time. Consequently, 95 per cent of the time the true difference in means will be contained between $3.9 - 2.12\ S.E._{\bar{x}_1 - \bar{x}_2}$ and $3.9 + 2.12\ S.E._{\bar{x}_1 - \bar{x}_2}$, i.e., between 3.9 − (2.12) (2.3) to 3.9 + (2.12) (2.3), or from −1.0 to 8.8.*

*Without reference to the t-tables, we often say that the true value should fall within 2 standard errors of the observed value, i.e., $3.9 \pm 2(2.3) = -0.7$ to 8.5.

Although the observed difference in means of 3.9 mg. was not significantly different from zero, we note from the confidence limits that the true difference in means readily could be as large as 8.8 mg. The investigator may use this confidence range to guide him as to what true difference might be feasible and important in a future, more definitive experiment. For example, the investigator may decide that a difference of 5.0 mg. in salivary ascorbic acid in favor of the caries-free group is important and, from the confidence range given previously, evidently feasible. Once he decides what true difference to focus upon, he can start to answer the question regarding sample size. An approximate solution to this question is given in the following discussion.

If the investigator is interested in detecting a true difference of at least 5.0 mg., he can determine how many cases the new study must include so that the observed difference between the means of the caries-free and caries-active groups ($\bar{x}_1 - \bar{x}_2$) is statistically significant from zero. If the true difference were zero, the normal curve of differences in means would be centered at zero with a standard deviation approximately

$$\sigma_{\bar{x}_1 - \bar{x}_2} = \bar{s} \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}.$$

Since it is always simpler to have the sizes of both samples the same, we shall put $N_1 = N_2$, so that

$$\sigma_{\bar{x}_1 - \bar{x}_2} = \bar{s} \sqrt{\frac{2}{N}}.$$

We are assuming that the value of $\bar{s}$ determined in the preliminary experiment will continue to be the average standard deviation of the readings in the two groups in the second, larger experiment. Furthermore, we are assuming that the second experiment will be large enough for the normal curve to be used instead of the t-curve.

The investigator must next decide whether he should use the 5 per cent or 1 per cent level of significance. He chooses the former if he is willing to take a chance five times out of 100 of saying that the true difference in means is not zero when, in fact, it is zero.* Let us assume that he chooses the latter because he would like to take this chance only once in 100 times.

If 5.0 mg. were the true difference, then, of course, the normal curve of differences in means would be centered at 5.0 mg. with a standard deviation

$$\sigma_{\bar{x}_1 - \bar{x}_2} = \bar{s} \sqrt{\frac{2}{N}}.$$

The investigator must decide how large a chance he wants to take of having the difference be not significantly different from zero, when, in fact, the true difference is 5.0 mg.† He may want to take this chance only five times in 100. This would insure that if the true difference is 5.0 mg., the observed difference will be significantly different from zero 95 per cent of the time. This is often called the power of the test.

*An "error of the first kind."
†An "error of the second kind."

The specific question then can be framed as, "If the true difference between the mean values of salivary ascorbic acid in the caries-free and caries-active groups is really 5.0 mg., how many cases should be studied in the new experiment so that the probability is 95 per cent that the observed difference in means ($\bar{x}_1 - \bar{x}_2$) will be significantly different from zero at the 1 per cent level?"

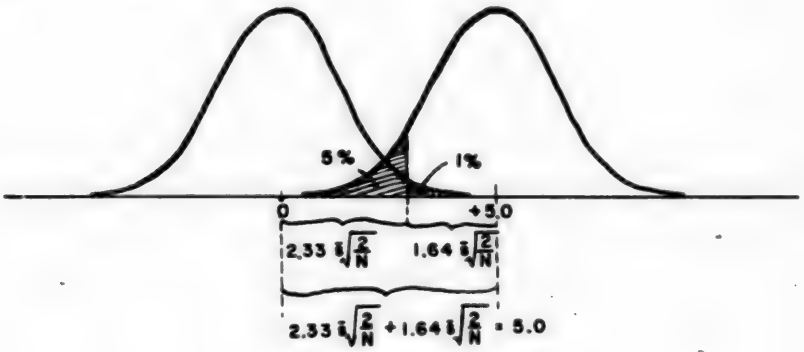

Fig. 1.

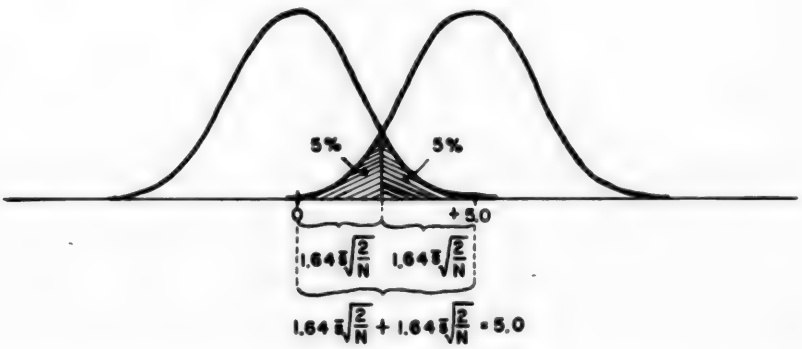

Fig. 2.

In the normal curve of differences in means centered at zero, the upper 1 per cent of the area of the curve is found to the right of $0 + 2.33\ \sigma_{\bar{x}_1 - \bar{x}_2}$. In the normal curve of differences in means centered at 5.0 mg., the lower 5 per cent of the area is found to the left of $5.0 - 1.64\ \sigma_{\bar{x}_1 - \bar{x}_2}$. The total distance between 5.0 mg. and 0 mg. thus must equal the sum of $2.33\ \sigma_{\bar{x}_1 - \bar{x}_2}$ and $1.64\ \sigma_{\bar{x}_1 - \bar{x}_2}$ (Fig. 1). Remembering that $\sigma_{\bar{x}_1 + \bar{x}_2}$ equals $\bar{s}\sqrt{\frac{2}{N}}$, we then have:

$$2.33\ \bar{s}\sqrt{\frac{2}{N}} + 1.64\ \bar{s}\sqrt{\frac{2}{N}} = 5.0$$

$$3.97\ \bar{s}\sqrt{\frac{2}{N}} = 5.0,$$

but $\bar{s}$ is taken to be 4.8 mg.,

$$(3.97)\ (4.8)\ \sqrt{\frac{2}{N}} = 5.0.$$

Solving this equation for N,

$$\sqrt{N} = \frac{(3.97)\ (4.8)\sqrt{2}}{5.0} = 5.39,$$

N = about 29 cases in each group would be necessary to show a statistically significant difference between the means under the circumstances stated above.

If the investigator desired to use the 5 per cent level of significance (Fig. 2), rather than the 1 per cent level, the equation to be solved would be:

$$1.64\ \bar{s}\ \sqrt{\frac{2}{N}} + 1.64\ \bar{s}\ \sqrt{\frac{2}{N}} = 5.0$$

and, solving for N, we find N = about 20 cases in each group.

If the investigator desires to proceed more carefully, he can use the 1 per cent level of significance in conjunction with a 99 per cent power of the test. The equation would then be:

$$2.33\ \bar{s}\ \sqrt{\frac{2}{N}} + 2.33\ \bar{s}\ \sqrt{\frac{2}{N}} = 5.0$$

and, solving for N, we find N = about 40 cases in each group.

It readily can be seen that the more conservative the level of significance chosen and the greater the power of the test used, the more cases would be necessary. If the value of the true difference desired were increased, it readily can be seen from the preceding equations that N would be smaller, and, similarly, if the value of the desired true difference were decreased, N would be larger. Table II presents the approximate size of the sample necessary in each group for significance to be asserted, corresponding to various values of the true difference in means at selected significance levels and powers of the test.

TABLE II

APPROXIMATE SIZE OF EACH SAMPLE NECESSARY TO ASSERT SIGNIFICANCE, ASSUMING $\bar{s}$ = 4.8 MG.

TRUE DIFFERENCE IN MEANS TO BE DETECTED	NUMBER OF CASES IN EACH GROUP		
	1 PER CENT LEVEL OF SIGNIFICANCE; 99 PER CENT POWER OF TEST	1 PER CENT LEVEL OF SIGNIFICANCE; 95 PER CENT POWER OF TEST	5 PER CENT LEVEL OF SIGNIFICANCE; 95 PER CENT POWER OF TEST
3.0	111	80	55 (69)*
4.0	63	45	31 (40)
5.0	40	29	20 (26)
6.0	28	20	14 (18)

*Values in parentheses were found by the method of Harris, Horvitz, and Mood.

The method just described affords a convenient way of approximating the sample size necessary for significance. It is approximate for several reasons: (1) the normal curve is the curve of reference instead of the t-curve, and (2) the average standard deviation obtained in the preliminary study is assumed

TABLE III

VALUES OF k*

$d_1 \backslash d_2$	1	2	3	4	5	6	8	12	16	24	32	∞
1	57.1	19.5	14.4	12.6	11.6	11.0	10.4	9.85	9.58	9.33	9.21	8.86
2	24.2	7.74	5.60	4.77	4.39	4.15	3.86	3.61	3.49	3.38	3.33	3.19
3	17.6	5.58	4.03	3.39	3.13	2.94	2.74	2.55	2.46	2.39	2.35	2.23
4	14.5	4.58	3.28	2.79	2.56	2.40	2.23	2.08	2.01	1.94	1.91	1.82
5	12.6	3.97	2.88	2.41	2.23	2.09	1.93	1.82	1.76	1.69	1.66	1.58
6	11.2	3.55	2.57	2.17	2.00	1.88	1.73	1.62	1.57	1.52	1.49	1.42
7	10.3	3.26	2.36	1.99	1.83	1.72	1.58	1.48	1.43	1.38	1.36	1.30
8	9.70	3.05	2.19	1.86	1.70	1.60	1.48	1.39	1.34	1.29	1.27	1.21
9	9.12	2.87	2.06	1.75	1.60	1.50	1.39	1.30	1.26	1.21	1.19	1.13
10	8.62	2.72	1.95	1.65	1.51	1.42	1.32	1.23	1.19	1.15	1.13	1.07
12	7.83	2.47	1.77	1.50	1.37	1.29	1.20	1.12	1.08	1.04	1.02	.971
14	7.22	2.28	1.63	1.38	1.26	1.19	1.11	1.03	.993	.959	.942	.893
16	6.73	2.13	1.52	1.29	1.18	1.11	1.03	.959	.924	.893	.878	.834
18	6.35	2.01	1.44	1.22	1.11	1.04	.972	.904	.872	.842	.828	.785
20	6.02	1.90	1.36	1.15	1.05	.991	.921	.858	.827	.798	.785	.744
25	5.37	1.70	1.22	1.03	.940	.884	.822	.765	.738	.712	.700	.663
30	4.89	1.54	1.11	.935	.855	.804	.748	.695	.671	.647	.636	.605
40	4.23	1.33	.962	.809	.739	.696	.646	.601	.580	.560	.550	.525
50	3.78	1.19	.854	.722	.661	.622	.577	.537	.518	.500	.492	.469
60	3.45	1.09	.778	.658	.602	.567	.525	.490	.472	.456	.448	.428
80	2.98	.940	.672	.569	.520	.490	.454	.423	.408	.395	.388	.369
100	2.67	.840	.600	.508	.465	.438	.405	.378	.365	.353	.347	.329

*Adapted from Harris, Horvitz, and Mood with the permission of the *Journal of the American Statistical Association.*

to remain the same for the second, larger experiment. The effect of this approximation is to make the estimated sample size somewhat small. The need for these assumptions has been removed, to a great extent, by a method derived by Harris, Horvitz, and Mood.[3] They present detailed tables for a significance level of 5 per cent with the power of the test either 80 per cent or 95 per cent. We will illustrate their technic, using the 95 per cent power.

Using the data presented in Table I, we wish to estimate the size of the sample necessary for significance to be asserted at the 5 per cent level, with a power of 95 per cent, when the true difference in means ($\bar{x}_1 - \bar{x}_2$) of 5.0 mg. is to be detected. The ratio,

$$k = \frac{5.0}{2\bar{s}} = \frac{5.0}{(2)(4.8)} = 0.521,$$

is obtained first.

On the horizontal axis of Table III, below d_1 (degrees of freedom of the preliminary sample) = 16, we find that a value of k = 0.521 falls between k = 0.580 (corresponding to $d_2 = 40$) and k = 0.518 (corresponding to $d_2 = 50$), so that d_2 is about 50.

Thus, the number of degrees of freedom of the second sample would be 50. Since the degrees of freedom in such a problem as we are studying is $N_1 + N_2 - 2$, and N_1 has been put equal to N_2,

$$2N - 2 = 50$$

so that the size of each sample would be $N = \frac{50 + 2}{2} = 26.$

For values of d_2 greater than 100, a method of interpolation is needed. Thus, for a true difference of 3.0 mg. we calculate,

$$k = \frac{3.0}{2\bar{s}} = \frac{3.0}{(2)(4.8)} = 0.313$$

which is smaller than the lowest k value (0.365) found under the column $d_1 = 16$, so that d_2 must be larger than 100 in this case. d_2 may be computed as follows:

$$d_2 = 100\,(K/k)^2$$

where K is the value in Table III for $d_2 = 100$ in the column headed by d_1. In the case of k = 0.313,

$$d_2 = 100\,(0.365/0.313)^2 = 136.0.$$

The size of each sample therefore would be:

$$N = \frac{d_2 + 2}{2} = \frac{136 + 2}{2} = 69.$$

The values of the second sample size obtained by the method of Harris, Horvitz, and Mood[3] are found within parentheses in the last column of Table II.

CORRELATED SAMPLES

Another type of experimental design which has wide application in dental research is that in which measurements of two related samples are compared. For example, two different technics may be used to measure the same character-

istic of one sample of individuals, or the same characteristic may be measured in one group at two different times. A frequent application of this latter type is the so-called before and after design, in which a sample is studied for the same characteristic before and after a certain treatment is applied. Thus, for example, we can take the problem in which the erythrocyte sedimentation rate is obtained in a group of individuals with periodontal disease, before and after surgical eradication of the periodontal pockets (Veatch and Young).[4] Because of the time-consuming aspects of this treatment, a preliminary study might be performed first and the readings tabulated for each group (Table IV).

TABLE IV

ERYTHROCYTE SEDIMENTATION RATES (MM./HR.) OF NINE PATIENTS WITH PERIODONTAL DISEASE, BEFORE AND AFTER SURGICAL ERADICATION OF THE PERIODONTAL POCKETS (VEATCH AND YOUNG)*

PATIENT NUMBER	BEFORE SURGERY (X_1)	AFTER SURGERY (X_2)	DIFFERENCE ($X_1 - X_2$) (Δ)
1	10	15	-5
2	8	3	+5
3	6	2	+4
4	9	5	+4
5	11	4	+7
6	23	15	+8
7	13	4	+9
8	11	8	+3
9	2	10	-8
	$\Sigma x_1 = 93$	$\Sigma x_2 = 66$	$\Sigma\Delta = +27$
	$\bar{x}_1 = 10.3$	$\bar{x}_2 = 7.3$	$\bar{\Delta} = +3.0$

*These data have been taken for illustrative purposes only, and any conclusions drawn do not necessarily pertain to the results of the original study as a whole.

The simplest way of taking advantage of the fact that the readings before periodontal surgery (x_1) are correlated with those after surgery (x_2) because the same group of patients is studied, is to take the difference in readings for each individual (Δ). These individual Δ values are summed, and the average difference ($\bar{\Delta}$) is found to be $\frac{\Sigma\Delta}{N} = \frac{+27}{9} = 3.00$ mm. This value of $\bar{\Delta}$ is, of course, equal to the difference between the average "before" reading ($\bar{x}_1 = 10.3$) and the average "after" reading ($\bar{x}_2 = 7.3$).

The t-test for statistical significance then proceeds as follows:

$$s_\Delta = \sqrt{\frac{\Sigma\Delta^2 - \frac{(\Sigma\Delta)^2}{N}}{N-1}} = \sqrt{\frac{349 - \frac{(27)^2}{9}}{8}} = \sqrt{33.50}$$

$$s_\Delta = 5.79$$

$$s_{\bar{\Delta}} = \frac{s_\Delta}{\sqrt{N}} = \frac{5.79}{3} = 1.93$$

$$t = \frac{\bar{\Delta}}{s_{\bar{\Delta}}} = \frac{3.00}{1.93} = 1.55,$$

where the sign of $\bar{\Delta}$ is not taken into account.

From standard tables of t at N − 1 = 8 degrees of freedom, we find that $t_{10\%} = 1.86$ and $t_{20\%} = 1.40$. Thus, the value of t = 1.55, which we have found, can be exceeded by chance factors alone between 10 per cent and 20 per cent of the time so that the average difference in E.S.R. before and after periodontal surgery is statistically not significant.

The investigator might feel, however, that further readings might produce a statistically significant result. In general, the E.S.R. usually drops after the elimination of infection. In fact, examination of the data in Table IV discloses that in seven out of the nine cases studied, there was a reduction in the E.S.R. after surgery. The question then arises: if further experimentation were to produce a sizable reduction in the E.S.R. after periodontal surgery, how many cases would be needed to demonstrate the statistical significance of this reduction?

Having the information from the preliminary study, we can proceed in a manner only slightly different from that employed with independent samples. Again, we may set up confidence limits around the observed average difference ($\bar{\Delta} = 3.00$) to guide us as to what average reduction it is feasible to expect. For 8 degrees of freedom we find that a value of t = 2.31 will be exceeded 5 per cent of the time through the action of chance factors alone. Accordingly, the 95 per cent confidence range is $3.00 - 2.31\, s_{\bar{\Delta}}$ to $3.00 + 2.31\, s_{\bar{\Delta}}$, or from −1.46 to 7.46.

The investigator may feel that it is worth while to detect a true difference of 3.00 mm. If the true average difference were 3.00 mm., how many cases would be necessary in order that there should be a 95 per cent probability that the observed average difference would be statistically significant from zero at the 1 per cent level? Since the upper 1 per cent of the area of the normal curve centered at a zero difference falls to the right of $0 + 2.33\left(\frac{s_\Delta}{\sqrt{N}}\right)$, and since 95 per cent of the area of the curve centered at 3.00 mm. falls to the right of $3 - 1.64\left(\frac{s_\Delta}{\sqrt{N}}\right)$, we have: $2.33\left(\frac{s_\Delta}{\sqrt{N}}\right) + 1.64\left(\frac{s_\Delta}{\sqrt{N}}\right) = 3.00$

Solving, we find N to be about 59 cases.

TABLE V

APPROXIMATE SIZE OF THE SAMPLE NECESSARY TO ASSERT SIGNIFICANCE, ASSUMING $s_\Delta = 5.79$ MM. PER HOUR

TRUE AVERAGE DIFFERENCE TO BE DETECTED	1 PER CENT LEVEL OF SIGNIFICANCE; 99 PER CENT POWER OF TEST	1 PER CENT LEVEL OF SIGNIFICANCE; 95 PER CENT POWER OF TEST	5 PER CENT LEVEL OF SIGNIFICANCE; 95 PER CENT POWER OF TEST
3.00	80	59	40 (63)*
4.00	45	33	23 (36)
5.00	29	21	14 (26)

*Values in parentheses were found by the method of Harris, Horvitz, and Mood.

Table V presents the approximate size of the sample necessary for significance to be asserted in this experiment for selected values of the true average difference corresponding to various significance levels and powers of the test.

The method of Harris, Horvitz, and Mood is again applicable to the estimation of the size of the second study. The value of k in this case is found as:

$$k = \frac{\text{true } \bar{\Delta}}{s_{\Delta}}$$

Thus, in order to estimate the size necessary to assert significance at the 5 per cent level with a power of 95 per cent, with an anticipated true mean difference of $\bar{\Delta} = 3.0$ mm.,

$$k = \frac{3.0}{5.79} = 0.518 \text{ at } d_1 = 8.$$

This value of k is between that found (0.525) in Table III for $d_2 = 60$ and (0.454) for $d_2 = 80$. Interpolating, we find $d_2 = 62$. This is the number of degrees of freedom in the second, or larger, study. Since, in this type of experiment using correlated samples, the degrees of freedom equals N – 1, the number of cases in the second study would be $d_2 + 1$, or, in this illustrative case, $62 + 1 = 63$. The values of the sample size obtained by the method of Harris, Horvitz, and Mood are found within parentheses in the last column of Table V.

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CLINICAL COMPARISON AND EVALUATION OF SPECIFIC XYLOCAINE HYDROCHLORIDE AND PROCAINE HYDROCHLORIDE SOLUTIONS IN MANDIBULAR BLOCK ANESTHESIA

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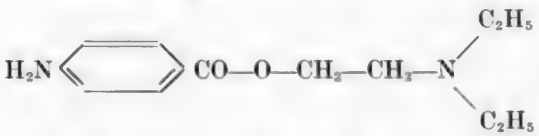
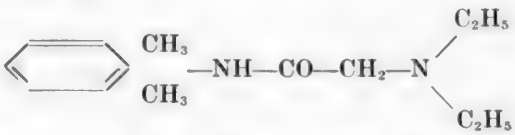
SINCE 1884, when cocaine was first utilized as a local anesthetic, there have been many attempts to synthesize substances having effective local anesthetic properties and a minimum of toxic effects. Procaine (Novocain) was introduced in 1905 and has the following structural formula:



In 1937, Erdtman and Lofgren showed that a series of acetanilid derivatives displayed local anesthetic faculties. In the course of continued investigation of substances possessing local anesthetic properties, Lofgren and Lundquist in 1943 synthesized omega diethylamino-2.6- dimethylacetanilid called Xylocaine or lidocaine, and indicated that this compound might be of clinical importance.[3] Structurally Xylocaine is:



According to Goldberg[3] and Gordh, this new anesthetic, unrelated chemically to cocaine, procaine, or procaine derivatives, was found to be (1) far more effective than other anesthetics of corresponding toxicity; (2) not irritating to the tissues in concentrations used in clinical work; (3) effective, both in topical application and for infiltration and conduction anesthesia; (4) an inherent vasoconstrictor and compatible with epinephrine; (5) chemically a very stable substance, e.g., able to withstand autoclaving, even in highly alkaline or acid solutions; and (6) it has been shown that Xylocaine with epinephrine shows the same systemic effect on respiration and blood pressure as procaine. Gruber[4] also stated that Xylocaine seems to be more selective for sensory and parasympathetic fibers and less specific for motor nerves.

The comparative toxicity of 2 per cent solutions of procaine, Monocaine, and Xylocaine has been given as follows:[6] procaine 2, Monocaine 2.66, and Xylocaine 4.2. If, however, the higher anesthetic efficiency of Xylocaine is

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considered the relative toxicity in infiltration and block anesthesia is considerably lower than that of procaine.[5]

According to Björn and Huldt,[1] at least the same anesthetic power as displayed by a 2 per cent procaine solution with adrenalin 1:40,000 may be effected by a 1 per cent Xylocaine solution containing only 1:100,000 adrenalin. The possibility of using a lower adrenalin concentration should be valuable in handling cases where a usual concentration would be dangerous. Lofgren[5] stated that in block anesthesia a 2 per cent solution of Xylocaine without epinephrine gave an anesthetic duration of 20 to 30 minutes. In a series of tests performed by me to determine the pH and osmotic pressures of the solutions used, it was found that the pH of the procaine solution used averaged 7.9, and the osmotic pressure averaged 4.1 atmospheres. In this same series, the Xylocaine solution used was found to have an average pH of 4.1 and an osmotic pressure of 8.9 atmospheres.

MATERIALS USED

The purpose of this investigation was to compare the clinical value, in inferior alveolar nerve anesthetic block injections, of a solution containing 2 per cent procaine hydrochloride and 1:40,000 epinephrine (pH 7.9) with a solution containing 2 per cent Xylocaine hydrochloride and 1:100,000 epinephrine (pH 4.1).

METHOD OF STUDY

The subjects for this work were 300 patients who came to the Clinic of Northwestern University Dental School for minor oral surgery. All cases selected required mandibular injections and were chosen at random in order to obtain an average cross section of patients.

To eliminate possible prejudice in recording results procaine and Xylocaine solutions were used on alternate days, and the operator was not informed of which agent was being tested. A nurse recorded the dates on which each solution was used, and this account was transferred to the results when the data were compiled for statistical evaluation.

Two cubic centimeters of the solution was used for each mandibular injection. This solution was injected at the rate of approximately one cubic centimeter every 30 seconds, and the time was recorded immediately upon the completion of the injection. The time interval between completion of the injection and onset of anesthesia was determined according to subjective symptoms of the patient, the criteria being the first noticeable change the patient could detect in the lower lip. Every 15 seconds thereafter, a probe was pressed against the soft tissues over the lower canine area on the side of injection. When the patient stated that the pressure of the probe no longer caused tenderness and that he felt only pressure, the time was recorded as that time of the surgical stage which would allow operating. After this, additional injections, such as for the buccinator nerve or midline anastomoses, were given, using one cubic centimeter of solution.

The patient's pulse rate, in a 15 second count, was taken immediately before injection, 3 minutes after completion of the injection, and again 15 minutes after the injection.

Surgery was begun as soon as possible after the surgical stage was recorded, and, by questioning the patient, the efficiency of the anesthetic was determined. If the patient experienced no pain at the time of operation, the efficiency was recorded as profound. If the patient experienced a little pain, but it was not great enough to warrant reinjection, the injection was considered fair. If enough pain was present to warrant reinjection, the injection was called a failure.

The amount of hemorrhage was noted at each operation and recorded as slight, moderate, or heavy. A slight hemorrhage was recorded if the socket had not filled with blood at the time of dismissal, moderate if some bleeding had taken place and a clot established at or before the time of dismissal, and heavy if bleeding was sufficient to interfere with ease of surgical procedure and in excess of what normally would be anticipated in an operation of the type performed.

During the period the patient was in the operating chair, the patient was observed for the following subjective symptoms: prolonged hyperpnea, change in color of the face, incidence of tremors in the limbs or jaw, amount of perspiration, or fainting. In cases where a change in subjective symptoms was observed after the injection, a record was made of these changes.

A self-addressed postal card was provided each patient and he was instructed to record the time, as accurately as possible, when sensations in his affected lip returned to normal, and to return the card by mail.

RESULTS AND DISCUSSION

In this investigation, records were made for 146 patients receiving mandibular injections of the procaine solution, and 154 patients receiving mandibular injections of the Xylocaine solution.

It was found that the mean time required to elicit the lip symptoms indicating onset of anesthesia, was less for the Xylocaine solution (81.4 seconds) than for the procaine solution (107.9 seconds) by 26.5 seconds. A statistical evaluation of these two times by the "t" ratio gives a probability value of less than 0.01, indicating that there is less than one chance in 100 that this difference is due to coincidence. Fig. 1 gives a graphic comparison of the onset times of the two solutions.

The mean time required to produce the surgical stage of anesthesia was found to be greater for the procaine solution (355 seconds) than for the Xylocaine solution (278 seconds) by 77 seconds. The "t" ratio for the surgical stage times of these two solutions gives a probability of less than 0.01, which is similar to the value for the onset time. Fig. 2 gives a graphic comparison of the surgical stage times for these two solutions.

The mean number of minutes of duration is less for the procaine solution (144.4 minutes) than for the Xylocaine solution (198.8 minutes) by 54.4 minutes. The probability value for these two times indicates that there is

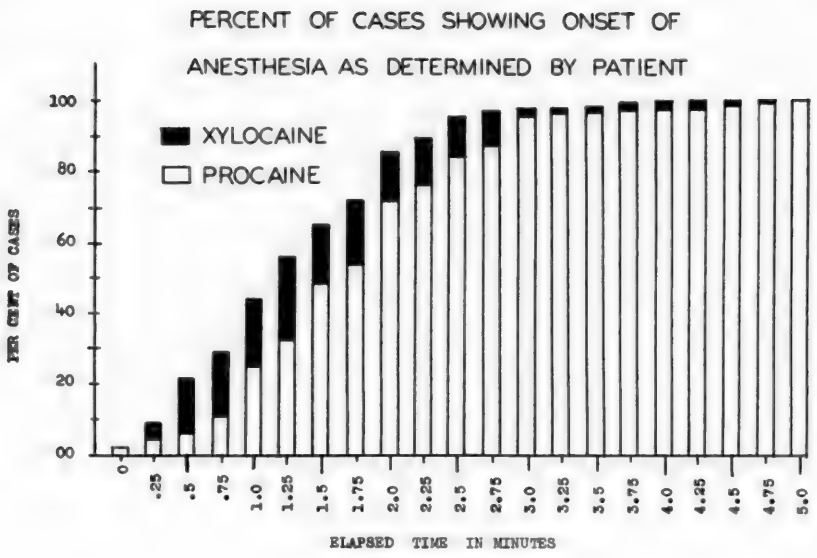

Fig. 1.

less than one chance in 1,000 that this difference is due to coincidence. When interpreting this difference, it is well to take note of the pH differences of the two solutions. Since the duration of procaine anesthesia is longer for acid than for alkaline solutions,[2] it might be assumed that if the pH of the two solutions had been more nearly the same, the difference in duration times would have been less. Fig. 3 gives a graphic representation of the duration times of the two solutions.

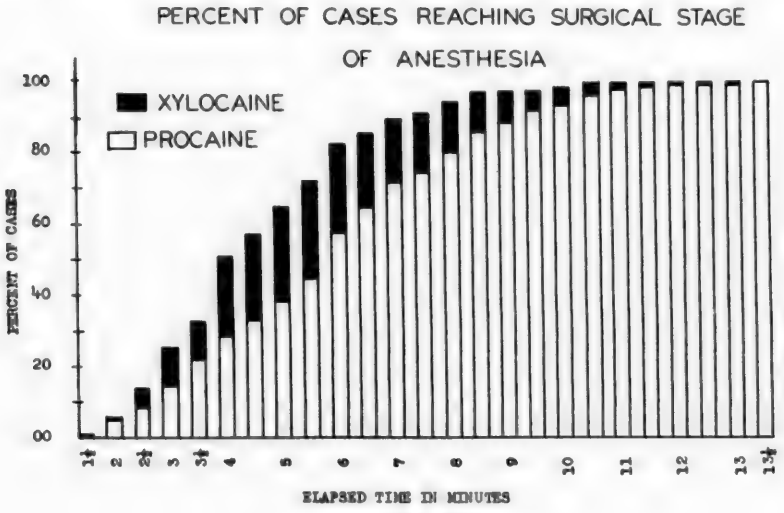

Fig. 2.

In this investigation it was found that 94.5 per cent of the procaine cases and 98 per cent of the Xylocaine cases showed profound anesthesia, 5.5 per cent of the procaine cases and 2 per cent of the Xylocaine cases were recorded as fair anesthesia. There were no cases of failure in either group. A statistical analysis of these data would indicate that there is no significant difference in the anesthetic efficiency of the two solutions for the particular injection used in this investigation.

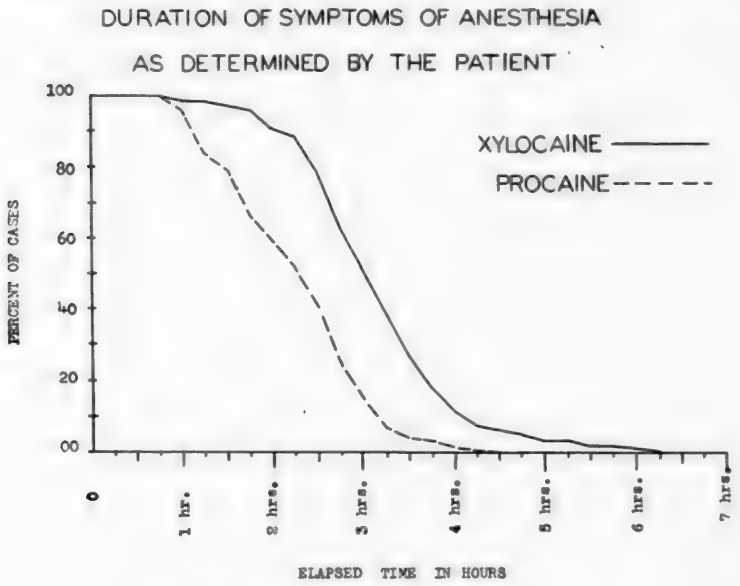

Fig. 3.

Table I gives an analysis of the change in the mean pulse rate of patients at the three time intervals when using the two solutions. It is of interest to note that the mean pulse rate before injection, for both groups, differs by only 0.07 of a beat per minute. This would tend to indicate that a very representative cross section of patients was obtained for both groups.

Statistical analysis of these rates reveals that the procaine solution caused a significant rise in the mean pulse rate at 3 minutes after injection, with a subsequent significant drop in pulse rate at the 15 minute interval. Thus, at 15 minutes after injection, there is no significant difference between the pulse rate then and that rate before the injection.

Xylocaine appears to cause no significant change in pulse rate at 3 or 15 minutes after injection. The drop in pulse rate between the 3 and 15 minute counts might be explained as the sum of the psychic attitude of the patient and the influence of the solution.

When it is considered that the procaine solution contains two and one-half times as much epinephrine as the Xylocaine solution, the different changes in pulse rates of the two groups is more readily understood.

TABLE I. CHANGES IN PULSE RATE

	PROCAINE SOLUTION (146 CASES)	XYLOCAINE SOLUTION (154 CASES)
Mean Pulse Rates		
Before injection	86.14	86.21
Standard deviation	14.02	12.40
3 minutes after injection	89.67	88.36
Standard deviation	14.79	13.43
15 minutes after injection	85.82	84.94
Standard deviation	13.01	11.98
"t" Ratio		
Rate before injection vs. rate 3 minutes after	2.090	1.466
Probability	0.04 to 0.05	No significance
Rate before injection vs. rate 15 minutes after	0.202	0.913
Probability	No significance	No significance
Rate 3 minutes after injection vs. rate 15 minutes after	2.36	2.35
Probability	0.05 to 0.01	0.05 to 0.01

The change in subjective symptoms occurring subsequent to the injection is recorded in Table II. Although it would appear that the Xylocaine solution caused fewer reactions, a statistical analysis of these data would indicate that there is no significant difference in the reactions of the two groups.

TABLE II. SYMPTOMS DURING ANESTHESIA

SYMPTOMS	PROCAINE SOLUTION (146 CASES)	XYLOCAINE SOLUTION (154 CASES)
Perspiration	7 (4.8%)	3 (1.9%)
Hyperpnea	3 (2.0%)	1 (0.65%)
Tremors	4 (2.7%)	3 (1.9%)
Pallor	3 (2.0%)	2 (1.3%)
Fainting	1 (0.68%)	0 (0%)

Table III shows the distribution of cases for the two solutions according to the amount of hemorrhage which was encountered during the operation. In order to compare this data statistically it was necessary to group the slight

TABLE III. HEMORRHAGE

	PROCAINE SOLUTION (144 CASES)	XYLOCAINE SOLUTION (153 CASES)
Slight hemorrhage	31 (21.5%)	37 (24.2%)
Moderate or normal hemorrhage	101 (70.2%)	112 (73.2%)
Heavy hemorrhage*	12 (8.3%)	4 (2.6%)
Chi square (X^2) of slight and moderate hemorrhage against heavy hemorrhage		4.695
Probability		0.01
Chi square (X^2) of moderate and heavy hemorrhage against slight hemorrhage		0.348
Probability		Not significant

*This includes two cases with procaine anesthesia in which it was necessary to give postoperative treatment in order to control hemorrhage. No such cases were recorded when using the Xylocaine solution.

and moderate hemorrhage cases for each solution and compare this total against the number of cases showing heavy hemorrhage. This grouping was made since, so far as surgical procedure is concerned, a slight or moderate hemorrhage offers no particular interference, while a heavy hemorrhage is considered undesirable. Statistical analysis revealed that the Xylocaine solution has a tendency toward less hemorrhage, or more local vasoconstriction, than does the procaine solution.

In view of the difference in epinephrine content of the two solutions, as mentioned previously, these data may be interpreted to indicate that some constituent of the Xylocaine solution, other than the epinephrine, also has vasoconstriction properties. This evidence would tend to substantiate the observations of Goldberg[3] and Gordh that Xylocaine produces some vasoconstriction.

CONCLUSIONS

Based on the evidence obtained in this investigation, the following conclusions were drawn:

1. The mean onset and surgical stage times are significantly shorter for the Xylocaine solution than for the procaine solution.

2. The mean duration time is significantly longer for the Xylocaine solution than for the procaine solution.

3. The Xylocaine solution caused significantly less change in the pulse rate three minutes after injection than does the procaine solution.

4. There is no significant change between the mean pulse rate before injection and the mean pulse rate 15 minutes after injection for either of the solutions.

5. Evidence indicates a slight tendency toward more hemorrhage when using the procaine solution than when using the Xylocaine solution.

6. There is no significant difference in the number of subjective reactions of the two groups.

7. There is no significant difference in the anesthetic efficiency of the two solutions for this particular injection.

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AN ELECTRONIC TECHNIC FOR RECORDING THE MYODYNAMIC FORCES OF THE LIP, CHEEK, AND TONGUE

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RECENTLY, at least five reports[1-5] have been submitted and as many devices have been constructed to measure the forces acting on the dental arch. All of these have pertained to the measurement of occlusal biting forces. Feldstein[6] employed the principle of hydraulics in a technic to measure the immediate forces on the buccal or lingual surface of a tooth.

The technic described herein has been proved sound in a laboratory study and, with refinements, is to be employed in a cross-sectional study of the University of Michigan High School and Elementary School children. It is capable of recording successively and accurately as many as seven pressures exerted by the lips, cheeks, and tongue upon various areas of the dental arch.

PHYSICAL PRINCIPLES INVOLVED

As the cross section of a carrying wire is decreased, its ability to carry an electrical current also is decreased, i.e., its resistance is increased. This principle has been utilized in a small filament of wire, the SR4 Baldwin Gauge, so that muscular pressure exerted on the gauge causes an elongation and changes the electrical conductivity of the wire.

Utilized in this capacity, a change in the force exerted produces a deformation of the gauge wire which can be plotted as a linear, straight line curve. Such a plotted curve indicates that one derives an output of voltage from the proper external measuring circuit which is proportional to the force applied. It then is possible to find the constant of proportionality at a point in the pressure range of the gauge, and this constant will maintain for any other point in the range. Any pressures outside the operating range will be so far from the linear constant that they will be easily detectable on the recorder. The gauges that were used registered a resistance within 0.3 ohm of each other.

For the external circuit the principle of the balanced Wheatstone bridge was used with modifications. A Wheatstone bridge has four wire arms with three known values and permits the finding of the fourth. In this technic the ratio arms are one to one, since the standard and the unknown are both wire gauges and within 0.3 ohm resistance of each other.

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In order to record successively the muscular forces acting on different areas of the mouth, the Wheatstone bridge which was used had been provided with this arrangement for switching between seven unknown gauges and a standard gauge. This arrangement was accomplished by the use of a scanning switch which included a motor to drive a cam wheel that activated the seven switches successively. Each switch had an unknown gauge balanced to the standard gauge. The balancing of each gauge was accomplished by a visual meter dial so that balancing was a quick, easy process. The balancer used was an Anderson Model 302 Bridge Balancer. In reality, therefore, there are seven Wheatstone bridges in one mounting, and all gauges are balanced to one gauge. A type of universal motor was used so that variation in the voltage to the motor secured a wide range of switching rates. Alternating current was used in the circuit because of the ease in amplifying the bridge voltage output. One thousand cycles per second current were used so that power line fields of sixty cycles per second would not interfere.

PREPARATION OF GAUGE

The gauges used are very sensitive and require protection as well as a definite means of insertion into the mouth. To meet this requirement a metal carrier, 2 mm. thick, 4 mm. wide, and 6 mm. long, was constructed with a soldered base and eyelets. The eyelets served as a means for attaching a rubber separator which went between the teeth and which held the carrier in position in the mouth. In cases where spaces are present, due to absent teeth, the metal gauge carriers are ligated to the teeth. On each width, on its inside surface, a half-round tube was soldered. Into one of these tubes half-round shafting, which consisted of a flat stainless steel spring wire soldered on its top surface, was fitted. Hence, the shafting, when inserted into the half-round tube, made a modified cantilever bridge with the free end of the spring wire resting on the top surface of the opposite half-round tube. On the bottom surface of the flat spring wire, in the center, the gauge was glued. The center of the top surface of the flat spring wire carried the plunger which received the muscular pressure. The spring and sides of the carrier were sealed off with a loosely fitting thin rubber sheet. Through one side of the carrier passed the leads which connected with the collecting box. The induced electromotive force thus continued through the gauge balancer to the scanning switch and then on to the gauge indicator and recorder. To take a reading the gauges were placed in position in the mouth and allowed to warm to mouth temperature. The muscles over each gauge in turn were held away from the gauge while it was being balanced to the standard gauge. This balancing procedure was accomplished visually by zeroing the indicator needle of the balancer with the standard gauge. When all gauges were balanced to the standard gauge and no muscular pressure was in action, it was possible to measure the muscle tonus of the relaxed musculature in the area of the measuring gauge. When measurement of a muscle action then was recorded, the variation in the two readings measured the force exerted by that particular motion.

The recording apparatus appropriate for this type of assembly was found to be that of the Brush Development Company which provided proportional readings of voltage as the applied pressure was increased. For the sample readings, a sound level recorder was used. The pressure on the gauge then became a logarithmic function of the balance point as recorded on the tape.

In the sample tracings that were made, two combinations of the gauges were used: one with five gauges on the labial and buccal surfaces, and a second group with four gauges on the labial and buccal surfaces and three on the lingual surfaces. The numbering of the gauges is shown in Fig. 1.

An analysis of the tracings indicates that the greater the depth of the recording, the smaller is the amount of pressure. The pointed dips in the tracings were due to the inertia of the recording stylus. Measurements for comparative studies and calibrations were made by measuring the length of the perpendicular as drawn in Fig. 2 for gauge No. 1.

The recordings in Fig. 2 were obtained while the patient was as relaxed as possible in order to secure the pressure of the oral musculature in a rest position. The patient's tongue was lying in the floor of the mouth away from the gauges on the lingual surfaces of the teeth. Under these conditions one obtains the long recording of the lingual gauges which signifies no pressure. At the same time the muscle pressure in the canine area of gauges 2 and 4 at rest, under the same conditions, produced a tracing similar to that of little or no pressure when compared to the recordings secured by tracings during the absence of lingual pressure. Gauge No. 1 showed a smaller recording than the others, signifying an increase of muscle force, and this increase in anterior gauge No. 3 was still greater as is shown by the greatly reduced tracing.

When the same individual was told to swallow, producing the subsequent increase of labial, buccal, and lingual pressure, Fig. 3 was secured. Gauge No. 1 recorded a greatly increased muscular force as revealed by the small amount of tracing in the second group of recordings. This increase exceeded that increase in the anterior gauge No. 3 so that now the pressure on the molar gauge exceeded that on the anterior gauge. The lingual tracings, as recorded, reveal the lingual pressure exerted in swallowing when compared with those of Fig. 2. The second series of tracings of gauges 5, 6, and 7 showed the beginning of relaxation of the tongue's musculature and a corresponding elongation of recordings. Fig. 4 was the recording of the labial and buccal forces under three different conditions. The first group of five recordings was secured when no labial or buccal pressure was being exerted. This recording was accomplished by holding the cheeks and lips away from the gauges that were in the mouth. The recordings under the title "No Pressure" show the typical long record. Gauge No. 5 of this series did not have all of the cheek muscle off the gauge and hence produced a shorter tracing than the other four tracings in the first group. The second group of five recordings included those of the relaxed, "at rest" musculature. Here the situation repeats itself, as seen in Fig. 2, with little or no change in the

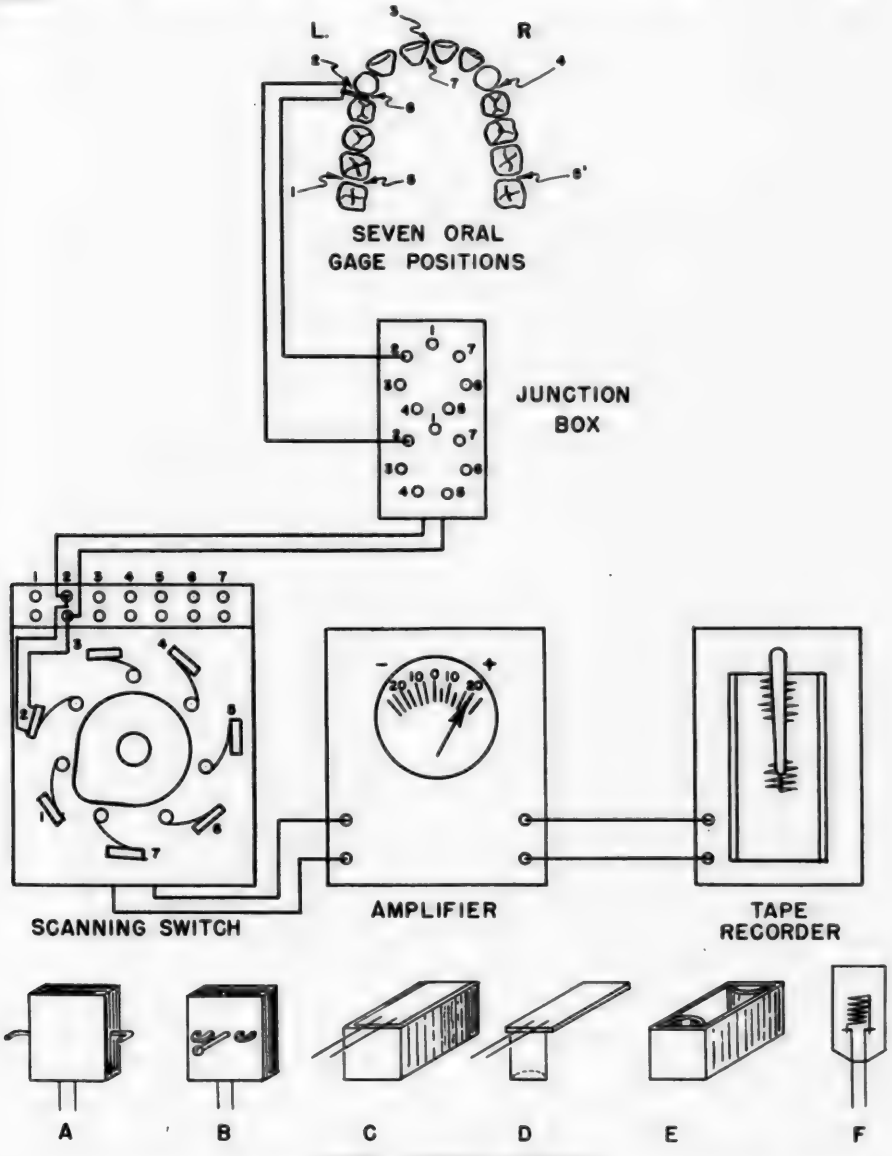

Fig. 1.—Electronic recording equipment arrangement. Top diagram shows positioning of the gauges in the mouth and connection to scanning switch with insulated cable by means of junction collecting box which has neck support for patient. Revolving cam wheel contacts individual G-RL micro switches which are connected to oral gauges and leads to the amplifier and recorder.

Gauge Carrier Details:

A, Gauge carrier with loops for ligation.

B, Gauge carrier with eyelets and elastic which is held in place by self-curing acrylic.

C, Spring in position in gauge carrier.

D, Spring soldered to post.

E, Gauge carrier showing two half-round tubes, one to receive one-half round shafting and other as support of free end.

F, Theoretical figure of SR strain gauge.

canine area, but with noticeable increase of force in the molar and incisor areas. The third group of recordings were those secured under the influence of muscle contraction during swallowing. Again the typical recording was determined of further increase in molar pressure exceeding that of the anterior increase, with the reaction of the cuspid area unchanged.

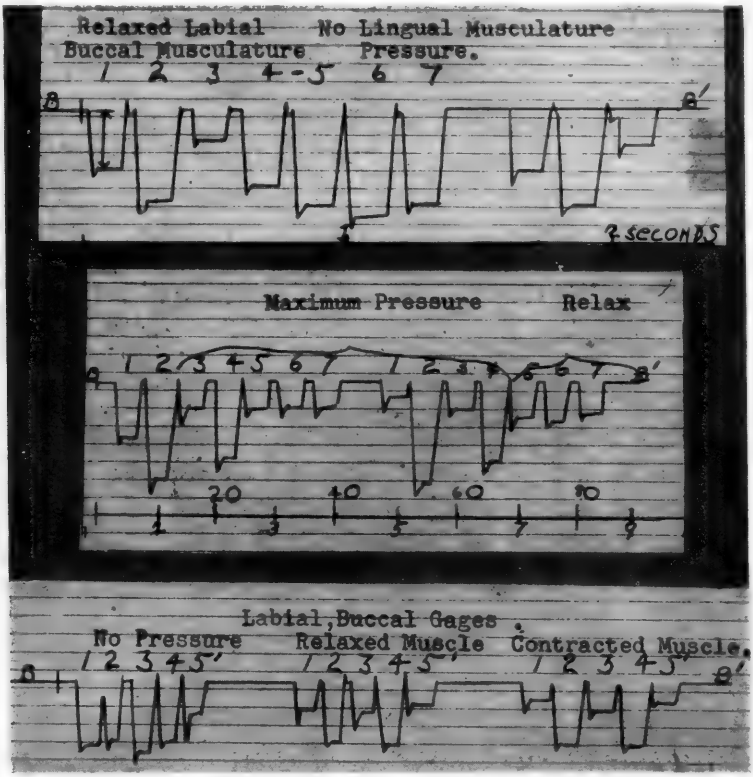

Fig. 2.

Fig. 3.

Fig. 4.

Figs. 2 to 4.

From the tracings which have been presented as evidence of the ability of the myodynamic recorder to register successively the muscular force exerted on seven areas of the oral cavity, no attempt has been made to assign interpretative values to the tracings in this introductory study. Hesitancy to do so arose from the fear that such values might be misinterpreted as definitive values for the different areas. This report, therefore, merely presents a technic by which the myodynamic recorder can be utilized to make an accurate study of the oral forces.

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Erratum

In the article by Viktors Kalnins, "The Effect of X-Ray Irradiation Upon the Mandibles of Guinea Pigs Treated With Large and Small Doses of Ascorbic Acid," which appeared on page 177 of the April issue of the JOURNAL, the second sentence in paragraph 3 read, "Furthermore, physiologic pharmacodynamic doses of vitamin C, exceeding several times the dose of ascorbic acid, act as detoxicants." This sentence should have been, "Furthermore, pharmacodynamic doses of Vitamin C exceeding several times physiologic dose of ascorbic acid, . . ."

LOWER FIRST PREMOLARS

Part I. A Definition and Classification of Discrete Morphologic Traits

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THE advantages offered by dental studies in the fields of growth, race, nutrition, and palaeontology are well known. Because of their peculiar sequence and time of eruption, the teeth serve as convenient markers for stages of growth. Their sensitivity to environmental change over long periods of time and their structural ability to withstand erosive agents in the ground make them of primary importance in reconstructing phyletic animal lines and in tracing the course of evolutionary change. The fact that teeth, in their more detailed morphologic structure, reflect the results of isolation, inbreeding, hybridization, drift, and other phenomena responsible for the genetic composition of populations makes dental analysis of human groups of great significance in the identification and classification of races, the populations which have, over varying periods of time and to varying degrees, maintained endogamous breeding. The teeth, too, manifest certain of the states of physiologic disequilibrium which, either in the past or at the moment, ontogenetically speaking, have occurred or are occurring. Thus, clinically the teeth are of undisputed significance.

It often has been stated in the past, and constantly is reiterated at the present time, that the entire dentition, in its gross morphology, is governed strictly by the action of genes. Korkhaus (1930) stated that there never has been much doubt of the heritability of tooth form, but at the same time admits that very little is known concerning the details of the genetic mechanism. Montagu (1933) indicated that the dentition is entirely genotypically determined. Keeler (1935) was of the opinion that the individual teeth are under genetic control. Results of studies by Swiss investigators, Jöhr (1934) in particular, indicate the important role of heredity in dental caries. Recently Lasker (1950) pointed out the large number of dental traits "in which genetic factors are important and in which evolutionary significance is manifest," although in his paper, somewhat misleadingly entitled "Genetic Analysis of Racial Traits of the Teeth," he indicates that as yet there has not been any genetic analysis of these traits. "The time is ripe," he urges, "for a really significant interdisciplinary project on the inheritance of human dental characteristics."

High on Birdsell's (1949) list of phenotypic characters, the genetic analysis of which will lead, presumably, to the "solution of New World origins," are dental traits; he pointed out that "little systematic racial work has been done in this area to date."

Dahlberg (1949) stated, "Basic studies on dentition which may eventually enable us to discuss dental characters in similar terms (in terms of gene fre-

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quencies) are under way, but unfortunately, they are not advanced far enough. We are still limited to phenotypic frequencies" (p. 138).

Expounding the field concept as a theory of development, Dahlberg goes on to say, "Genes, of course, are assumed to regulate and determine the fields. Certain gene complexes are probably responsible for the fields which determine the dentition as a whole and the primary characters. Other genes would modify these fields as to strength, extent, etc., and produce the secondary characteristics" (p. 173).

In view of the fact that dental characters are acknowledged as being controlled genetically, Boyd (1950) could list only one dental trait among seventy-one human characteristics for which the "supposed genetic relationships of factors" are tentatively suggested. This trait, the hereditary absence of teeth (various types), is neither morphologic nor of common occurrence, and hence is of little use in population analysis.

The prelude to genetic analysis of phenotypic traits must be precise definition and classification. A survey of the literature reveals that a thorough study of common discrete dental traits has not been made yet. Numerous studies have been made with one or another objective in view, but none of these have been amenable to genetic analysis. Thus, while we have admirable studies of the number of molar cusps, topical arrangements of these cusps, Bolk's paramolar tubercle, the protostylid, shovel-shaped incisors, enamel pearls, etc., we find no statements as to the genetic factors of their inheritance, beyond the mere opinion that these traits are indeed inherited. The only exception is the study by Kraus (1951) of the inheritance of Carabelli's anomaly.

Perhaps the most recent appraisal of the status of morphologic studies of the dentition is that of Lasker (1950). We might classify those dental traits in which he considers "hereditary factors are predominant" as shown in Table I.

TABLE I

CLASSIFICATION	DENTAL TRAITS
Developmental	Order of eruption, age at eruption, etc.
Mensurable	Crown dimensions, root length
Morphologic	Crown pattern, wrinkles, cusplets, shovel-shaped incisors, lingual ridges, dental tubercles, enamel extensions, enamel nodules, cusp number, Carabelli's anomaly, fused roots, bent roots, taurodontism
Anomalous	Supernumerary roots, supernumerary teeth, congenitally missing teeth, peg teeth, paramolar tubercle
Topical	Diastemata, trema, bite and jut, shape of dental arches, prodontism

In addition he lists mandibular and maxillary hyperostoses, which are not strictly dental traits.

Our interest will center upon those traits classed as morphologic. With the exception of the Carabelli anomaly none of these traits have undergone genetic analysis. For example, Lasker points out that in spite of numerous detailed studies of molar crown pattern and cusp number "the inheritance of the number and pattern of cusps has not . . . yet been systematically traced in pedigree studies." Little or no genetic study has been made on enamel margins, exten-

sions, and nodules, traits which Pedersen (1949) observed and classified racially. With one possible exception, then, the numerous studies of dental characters which commonly occur, in one form of expression or another, in human populations have yielded no genetic interpretations.

Since, at the present time, we are aware of not more than ten loci on human chromosomes having to do with common phenotypic traits, i.e., blood groups and types, Rh factor, blood group antigen secretion, etc., it is a matter of great interest and importance that more human loci be defined and their genic potentialities and actions classified. With the identification of additional human genes, the road toward better understanding of the processes of human evolution, of race formation, and of breeding will be laid open. The classification of human populations, from the familial through the subspecific level, will be placed upon a more refined and sensitive basis. With human groups classified according to the relative frequencies of a large number of genes, we can hope for more accurate reconstruction of racial history.

The research of which this article is the first expression has as its goal three major tasks: (1) the reduction of the human dentition to a number of discrete morphologic traits of common, not rare, occurrence in populations; (2) proving where possible that these traits are governed strictly by the laws of inheritance; and (3) indicating the precise mode of inheritance whereby these traits are manifested phenotypically.

As an initial step we propose to define seventeen morphologic traits located on the lower first premolar.

LOWER FIRST PREMOLAR

After a preliminary survey of the literature and an independent study of large samples of each type of tooth, it was decided to begin our research with the lower first premolar. This tooth shows an extremely wide range of morphologic variability, is less inclined toward occlusal wear because of the inclination of its masticatory surface, and apparently has not been subjected to much scrutiny in past dental studies.

Hrdlicka (1921) has brought conveniently together the results of studies prior to 1920 of the morphologic characteristics of lower premolars. Hrdlicka concluded that, ". . . these ridges and depressions . . . have not as yet been studied sufficiently intensively and comprehensively. It is seen that as a result these formations are still known but imperfectly; that their significance has not as yet been clearly established; and that in some cases they have received a wrong interpretation."

Black (1894) described the morphology of the lower first premolar, based upon a rather limited sample, without any attempt at classification. Wortman (1886) described the lower premolars in general, while Zuckerkandl's observations (1891) were extremely elementary and it is doubtful if he had in mind a classificatory inquiry.

Mühlreiter (1912) likewise described both premolars in very general stereotype fashion, not even recognizing a size variation in the median ridge.

By far the most thorough morphologic description of lower first premolars was undertaken by de Jonge-Cohen (1920). His chief concern, however, was with the variation in pattern of the sulci of the occlusal surface of the tooth. A follower of Bolk, in his adherence to the dimer theory, de Jonge-Cohen considered the basic premolar type as a sexitubercular tooth possessing three buccal and three lingual components. From this basic type a quintitubercular form develops, lacking the mesiolingual element. All premolar variants from these two forms are to be understood as developments from them, and hence can be classified as belonging to the one or the other basic type. De Jonge-Cohen lists four factors that may bring about forms which are strongly deviant from the original types: The relatively slight development of accessory cusps, strong development of the margins, the degree of development of the mesial crown margin, and the relatively slight development of the lingual cusp. Although the elements for a first-class morphologic description are present in de Jonge-Cohen's paper, he concluded with a classificatory scheme based upon a bipolar developmental history. In general, the author is occupied with the thesis of causation and he leans heavily upon the role of function in the formation of premolar structure. Had he surveyed an extensive sample of teeth and noted the wide range of variation, his functional formulations would have been offered with much less assurance.

More recently Jackson and Blount (1942) stated categorically that the premolar cusps are united by a median ridge so that "the masticating surface presents two small pits." This, as will be shown, is by no means the situation in numerous cases.

Because of the lack of definitive criteria by which to classify lower premolars, Pedersen (1949) was constrained to say that "as accurate criteria are difficult to establish, the present writer made no attempt to calculate the incidence of the cuspid type of Pl inf. in the East Greenland Eskimo." As far as the crown is concerned, Pedersen observed only the number of cusps, which in Eskimos appears to be two as a general rule. Three cusps, he claimed, are rarely observed. "The general pattern of premolar crowns," he pointed out, "also seems to be largely the same in Eskimo and Whites, in so far as cusp numbers and arrangement of the cusps are almost identical."

With the lack of definitive criteria, it becomes apparent that racial classification and genetic analysis based on premolar teeth are considerably hampered, if not rendered impossible of accomplishment.

We began our study by drawing freehand over 100 lower premolars, both right and left, observed in five aspects: buccal, mesial, lingual, distal, and occlusal. Various morphologic structures of this tooth were differentiated on a twofold basis; structures which showed considerable variation were classed separately from those manifesting little, if any, deviation. On this basis seventeen traits eventually were isolated and defined. The variations of each of these traits were then observed on over 800 right and left lower first premolars, and their frequencies were recorded.

The premolars comprising the data for this study were derived from several ethnic groups: Papago Indians, Caucasoids, Yaqui Indians, Mexicans,

Chinese, and Negroes. Dental casts of these groups were collected over a two-year period in southern Arizona. In addition, a small series of unerupted lower first premolars extracted from the mandibles of prehistoric Arizona Indian skeletons were utilized as a control for estimating the amount of change brought about by wear. The Caucasoid series includes both casts and extracted teeth obtained from Tucson dentists. Comparison of the results obtained from casts, on the one hand, and the extracted teeth on the other, indicated that casts were not suitable for accurate recording of certain traits. These traits were eliminated from consideration. Other traits were eliminated on the basis of their rare occurrence in all but one form. It should be borne in mind that the purpose of the study was to define only traits of common occurrence in human populations.

During the course of the study, certain possibilities extraneous to our immediate aims suggested themselves. One was the distinct probability that in the analysis of dental morphology lies a more accurate diagnostic tool for twin zygosity than any yet devised, including histologic study of the afterbirth. This, of course, is generally in accord with Newman's (1937) opinion. The second was the possibility that in the analysis of the dental genotype there exists the basis of a new method of identification, including positive as well as negative results.

DEFINITION OF TRAITS

External Lingual Grooves.—An external lingual groove is a groove that extends any vertical distance inferiorward on the lingual aspect of the tooth. There may be no groove present, in which case the lingual aspect of the lingual cusp (deuteroconid) presents a smooth appearance (Fig. 1). A single groove may be present (Fig. 2), or two lingual grooves may be present (Fig. 3). The presence of three lingual grooves has not been established clearly in over 1,000 premolars examined for this characteristic.

Sagittal Sulcus.—The main (or sagittal) sulcus runs in a mesiodistal direction between the buccal (protoconid) and lingual (deuteroconid) cusps. Two different aspects are presented. The sulcus may be complete, that is, the fissure is uninterrupted and runs continually and at a more or less constant depth mesiodistally. It may, on the other hand, be interrupted or "dammed up" by one or more extrusions of protoconid or deuteroconid ridges.

Relative Position of the Deuteroconid.—This trait concerns the relationship of the main mass of the deuteroconid to the long axis of the central protoconid ridge. Three positions are noted:

1. If the bulk of the deuteroconid is situated anterior to the long axis of the central protoconid ridge, the position is described as *mesial* (Fig. 4).

2. If the bulk of the deuteroconid is situated posterior to the long axis of the central protoconid ridge, the position is described as *distal* (Fig. 5).

3. If the body of the deuteroconid is bisected by the long axis of the central protoconid ridge, the position is described as *median* (Fig. 6).

It is important in observing this trait to orientate the premolar so that the lingual aspect of the tooth is presented at right angles to the mesiodistal plane of the tooth, without reference to its position in the dental arch.

Number of Lingual Cusps.—The central lingual cusp is termed the deuteroconid. Accessory cusps may be present, and it is often difficult to ascertain in some cases which can be designated the deuteroconid. It has been the practice

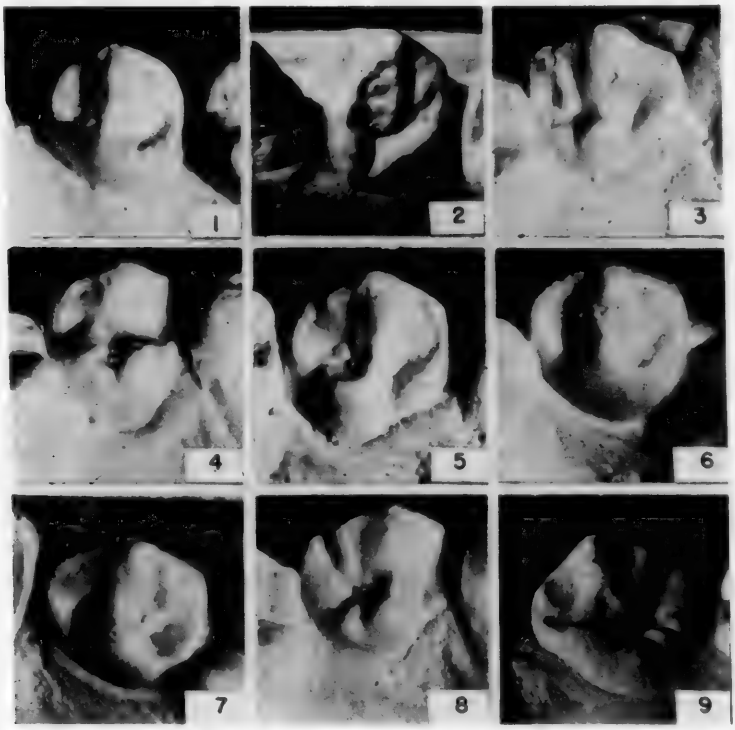

Fig. 1.—Absence of external lingual grooves (right).
Fig. 2.—A single external lingual groove (right).
Fig. 3.—Two external lingual grooves (left).
Fig. 4.—Mesial position of the deuteroconid (left).
Fig. 5.—Distal position of the deuteroconid (left).
Fig. 6.—Median position of the deuteroconid (right).
Fig. 7.—One lingual cusp (right).
Fig. 8.—Two lingual cusps (left).
Fig. 9.—Five lingual cusps (right).

in this study, where two or more lingual cusps or cusplets are present, to regard that cusp showing the closer relationship to the protoconid (directly connected to the protoconid ridge) as the deuteroconid. Consistency on this point is important for classifying the position of the deuteroconid. A lingual cusp is defined as having an independent apex, however slight. The deuteroconid is considered a lingual cusp, even though it may be fused with the protoconid. Hence, by definition there is always at least one lingual cusp per premolar (Fig. 7). There may be two (Fig. 8), three, or four lingual cusps. Only one example of five lingual cusps has been observed in our material (Fig. 9).

Mesial Protoconid Margin.—By definition, the mesial protoconid margin includes that portion of the margin of the tooth mesial to the apex and extending down along the mesial edge of the tooth. The margin in general presents

either of two aspects. There may be a ridge bordering all or part of the mesial margin, as viewed from the lingual aspect. The ridge may be confined only to the occlusal margin, or only to the mesial margin. It may be broad or narrow, marked or not by an indentation on its inner side (Fig. 10). There may be no ridge present anywhere along the mesial protoconid margin (Fig. 11).

Mesial Deuteroconid Margin.—The deuteroconid, as defined previously, may be connected directly with the mesial margin of the protoconid by a ridge marked on its inner aspect by a furrow or sulcus. The ridge may appear as a continuation of the mesial protoconid marginal ridge, or it may be present when the mesial protoconid marginal ridge is completely absent. Conversely, the latter may be present when there is no mesial deuteroconid marginal ridge. If the ridge is cut through anywhere along its length by a lingual groove, it is regarded as absent. In other words, the ridge connecting the deuteroconid with the mesial protoconid margin must be continuous. Fig. 12 illustrates a mesial deuteroconid margin ridge continuous with the mesial protoconid marginal ridge. Fig. 13 shows the absence of the mesial deuteroconid marginal ridge.

Distal Protoconid Margin.—As is the case with the mesial margin, the distal protoconid margin may display a ridge (Fig. 15). Usually the ridge, when present, is not as sharply defined as that which occurs on the mesial margin. Fig. 14 shows the distal protoconid margin without the ridge.

Distal Deuteroconid Margin.—The distal deuteroconid margin may possess the ridge under the same conditions as found with the mesial deuteroconid margin (Fig. 16), or it may lack the ridge entirely (Fig. 17).

Central Occlusal Ridge of the Protoconid.—On every tooth observed there was present, on the occlusal surface of the protoconid, a ridge running from the apex of the protoconid lingually to the base of the protoconid or to the adjoining deuteroconid. The ridge may possess a sharp edge with straight sides of equal breadth, forming in cross section nearly an equilateral triangle with its base formed by the occlusal surface of the protoconid, or it may have a rounded irregular surface, presenting contours of varying forms in cross section. In some cases the ridge takes a straight uninterrupted course for its entire length (Fig. 18), but in others it is bifurcated with another ridge leading off it and running obliquely mesial- or distalward down the lingual face of the protoconid (Fig. 19). The former might be termed an unbifurcated ridge, the latter a bifurcated ridge.

Number of Occlusal Protoconid Ridges.—While every lower first premolar observed by us exhibited the main protoconid ridge, there were some which had one or more additional ridges, either mesial or distal to the central ridge or on both sides of it. Cases where more than four occlusal protoconid ridges are present were extremely rare. Hence four categories are recognized:

1. One occlusal ridge (Fig. 20),
2. Two occlusal ridges (a bifurcated main protoconid ridge is considered as two ridges) (Fig. 21),
3. Three occlusal ridges (Fig. 22),
4. Four occlusal ridges.

Number of Occlusal Pits.—It was found that casts are not entirely reliable for recording the disposition or numbers of pits on the occlusal surface of the premolar, nor for tracing the course of minor sulci. Apparently the impression material used was too coarse to enter the more minute pit cavities. Although the number of pits was recorded in this study, only those observed on actual teeth may be considered as accurately recorded. When the numbers of pits recorded from casts were compared with the numbers found on extracted teeth,

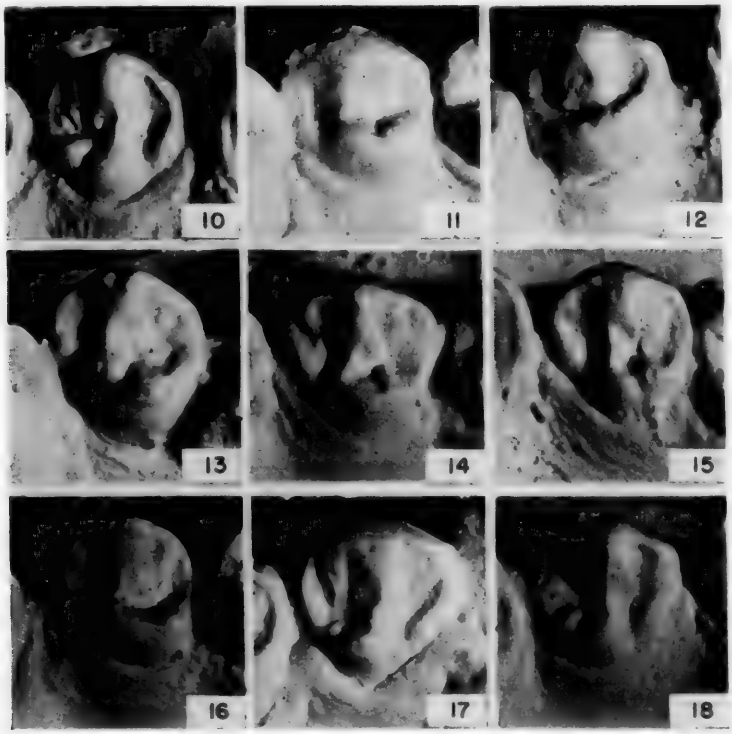

Fig. 10.—Mesial protoconid marginal ridge (left).
Fig. 11.—Absence of mesial protoconid marginal ridge (right).
Fig. 12.—Mesial deuteroconid marginal ridge continuous with the mesial protoconid marginal ridge (left).
Fig. 13.—Absence of mesial deuteroconid marginal ridge (right).
Fig. 14.—Absence of distal protoconid marginal ridge (right).
Fig. 15.—Distal protoconid marginal ridge (right).
Fig. 16.—Distal deuteroconid marginal ridge (right).
Fig. 17.—Absence of distal deuteroconid marginal ridge (left).
Fig. 18.—Unbifurcated occlusal protoconid ridge (left).

the latter by far predominated. Since in our estimation this trait may have great significance, both clinically and anthropologically, it is suggested that only actual teeth, showing neither dental fillings nor caries, be considered in classifying according to this criterion.

Deuteroconid-Protoconid Relationship.—The main lingual cusp, or deuteroconid, may be related morphologically to the protoconid in any one of several ways. Basically these may be resolved into two: fused or joined with the

protoconid, and independent of the protoconid. An independent deuteroconid is one whose apex is either completely free and can be palpated, or whose apex is united to the main protoconid ridge by means of a prominent horizontal ridge (Fig. 23). A fused deuteroconid is one whose apex is connected to the central protoconid ridge by a ridge sloping upward buccally, or whose apex is continuous or almost continuous with the lingual surface of the protoconid, in which case the deuteroconid appears in relief on the occlusal surface of the protoconid (Fig. 24).

Mesial Buccal Margin.—The mesial margin of the lower first premolar may be bounded on the buccal aspect by a ridge separated from the body of the tooth by a shallow vertical groove. This often may be seen only by casting a side light on the buccal surface. In the majority of cases this ridge and accompanying groove are absent.

Distal Buccal Margin.—As is the case with the mesial buccal margin, a ridge and accompanying shallow vertical groove may be present on the distal buccal margin. The distal ridge may be present without the mesial ridge being present, and vice versa. The absence of the ridge and groove is the more usual condition.

Plane of the Occlusal Protoconid Ridge.—When the occlusal plane of the mandibular dentition is confluent with the horizontal, the lingual border of the occlusal protoconid ridge forms an angle with this plane, ranging from a few degrees to 90 degrees. In some cases it is identical with the horizontal plane. As defined in this paper, the angle or plane of the protoconid ridge can be observed only in casts. This means that the lingual or buccal slope of the premolar is a factor in the determination of the inclination of the ridge. Therefore, in casts where the premolar is significantly out of line with the rest of the dentition, this observation is omitted. For this reason also, observations on extracted teeth were not taken, since orientation as defined herein could not be established. Three planes of inclination are recognized: vertical, oblique, and horizontal. The premolar must be observed in a plane established by the mesial and distal buccal margins; hence, it is almost impossible to take satisfactory photographs of this trait.

Size of the Protoconid Ridge.—This is subjectively determined. The effects of wear must be assessed, and the size of the protoconid ridge relative to that of the individual tooth is the basis for judging. The tooth is observed lingually and superiorly. If the ridge is broad and/or protruding prominently from the occlusal surface, it is classified as *prominent* (Fig. 25). If the ridge is neither broad nor prominent it is termed *slight* (Fig. 26).

Buccal Aspect of Coronal Profile.—When the buccal surface of the premolar is observed at right angles to the linguobuccal plane, the profile is classifiable as straight slightly convex, or prominently convex (Fig. 27). This determination can be made upon either casts or single extracted teeth. The profile is considered to embrace the entire crown surface, from protoconid apex to the neck of the tooth.

SUMMARY

1. The hereditary basis of the dentition long has been recognized.

2. With the exception of a single trait, Carabelli's anomaly, there has been no genetic analysis of common nonpathologic, morphologic dental characteristics.

3. The addition of genes governing dental features to the small pool of human genes for common phenotypic traits already established would be of great significance for furthering our understanding of the dynamics of human evolution, of race formation, and of population genetics.

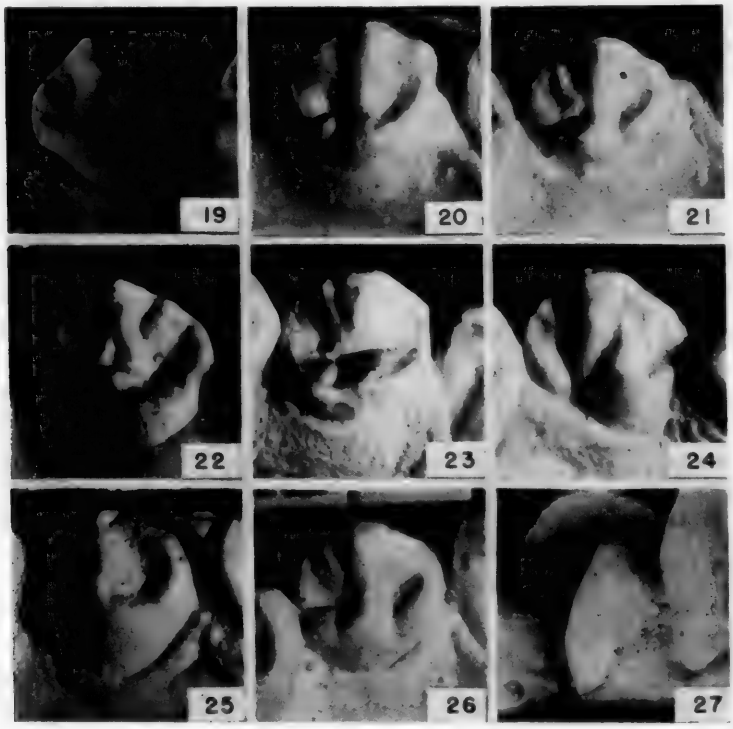

Fig. 19.—Bifurcated occlusal protoconid ridge (right).
Fig. 20.—One occlusal protoconid ridge (left).
Fig. 21.—Two occlusal protoconid ridges (left).
Fig. 22.—Three occlusal protoconid ridges (right).
Fig. 23.—Independent deuteroconid (left).
Fig. 24.—Fused deuteroconid (right).
Fig. 25.—Prominent protoconid ridge (left).
Fig. 26.—Slight protoconid ridge (left).
Fig. 27.—Prominently convex buccal coronal profile (right).

4. Isolation, definition, and classification of morphologic dental traits that are of common occurrence are necessary preliminary steps to genetic analysis.

5. The lower first premolar was selected for study because of its wide range of morphologic variability. Previous studies of this tooth have not provided data amenable to genetic analysis.

6. Seventeen traits, and their common varieties, have been isolated and defined for the lower first premolar, based on observations of more than 800 teeth, both on casts and extracted premolars, representing a number of ethnic groups.

The authors are deeply indebted to Mr. Robert Morrow, Superintendent of the Tucson Public Schools, for permission to obtain dental impressions of school children in the schools. The nurses and principals of the individual schools were most cooperative in placing their facilities at our disposal. The Most Reverend Daniel J. Gercke, Bishop of Tucson, very kindly provided access to the parochial schools in the southern Arizona area. Throughout the study Mrs. Bonnie Jones, anthropology graduate student, processed the thousands of observations. The research was supported financially in its earlier phases by a fellowship grant awarded to the senior author, Dr. Kraus, by the John Simon Guggenheim Memorial Foundation, and in its later phase by a grant and considerable photographic assistance from the Chicago *Daily News*. To both institutions the authors express their deep gratitude.

ADDENDUM

Although anticipatory of papers to follow the present one, it should be stated that the traits defined herein have been established as inheritable. Furthermore, it subsequently will be shown that each is inherited as an independent variable.

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PRENATAL INFLUENCES ON TOOTH DEVELOPMENT

II. Artificially Induced Fever in Rats

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AS INDICATED in a previous report,[1] little information is available regarding prenatal influences on tooth development. Although clinical observations have demonstrated such a relationship,[2, 3] experimental study may provide the means for gathering considerably more information on the etiology and pathogenesis of congenital dental abnormalities. Since it would be impossible to duplicate in experimental animals the variety of maternal disturbances regarded as etiologically significant, it would seem advisable to consider only those factors that might be common accompaniments of many such disease states. This type of experimental approach may be a valid one since it is conceivable that various common denominators of maternal disease may be the actual causative agents of so-called congenital dental defects.

Inasmuch as fever is an accompaniment of many disease states, it is possible that it alone may exert a significant influence on the developing oral structures. This has been established previously by animal experiments,[4-6] but little evidence is available that the same factor may be responsible for dental developmental abnormalities in the fetus in utero. Runge,[7] in a treatise on the etiology of abortion occurring during maternal disease, considered elevated temperature to be a primary cause of fetal death and far more significant than the infective agent per se. No mention, however, was made of pyrexia as an etiologic factor in congenital abnormalities.

MATERIALS AND METHODS

Thirty-three virgin Wistar strain albino rats were mated with males of proved fertility. Body weights ranged from 175 to 250 grams. A nutritionally adequate diet was fed to all animals. The onset of gestation was recorded as the day when spermatozoa were demonstrated in vaginal smears. The females then were segregated and their subsequent course of pregnancy was followed by weight.

Following conception, five animals were designated as controls and allowed to continue an uninterrupted course of pregnancy. The remaining twenty-eight animals were grouped according to trimesters of pregnancy and subjected to artificially induced fever by means of an incubator equipped with ventilation and humidity controls. Six rats were treated in this manner during their first trimester of pregnancy, ten during their second, and twelve during their third trimester. Rectal temperatures were taken of each animal prior to placing it in the incubator and again at hourly intervals during the

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TABLE I

ANIMAL NUMBER	INDUCTION OF FEVER: TRIMESTER OF GESTATION	INDUCTION OF FEVER: DURATION OF FEVER (HOURS IN INCUBATOR)	INDUCTION OF FEVER: RECTAL TEMPERATURE (DEGREES FAHRENHEIT): PRIOR TO ENTERING INCUBATOR	INDUCTION OF FEVER: RECTAL TEMPERATURE (DEGREES FAHRENHEIT): DURING PERIOD IN INCUBATOR: AVERAGE	INDUCTION OF FEVER: RECTAL TEMPERATURE (DEGREES FAHRENHEIT): DURING PERIOD IN INCUBATOR: MAXIMUM	INDUCTION OF FEVER: INCUBATOR TEMPERATURE (DEGREES FAHRENHEIT): DRY BULB	INDUCTION OF FEVER: INCUBATOR TEMPERATURE (DEGREES FAHRENHEIT): WET BULB	TERMINATION OF PREGNANCY: DIED (HOURS AFTER ONSET OF FEVER)	TERMINATION OF PREGNANCY: DELIVERED (DAYS OF GESTATION)	YOUNG: LIVE BIRTHS	YOUNG: STILL-BIRTHS	YOUNG: FETUSES PRESENT AT DEATH
1	1	12	98.0	103.3	103.8	102.2	90.1		23	10	0	
2	1	24	98.4	102.1	103.4	100.2	93.0		22	13	0	
3	1	24	99.0	104.2	106.4	100.0	91.0		23	5	0	
4	1	48	99.6	103.1	103.6	99.3	88.4		25	4	0	
5	1	48	99.0	103.3	103.8	99.9	89.8		21	11	0	
6	1	48	98.8	102.5	103.8	100.0	90.2		? abort.			
7	2	11	98.9	103.5	104.1	100.0	92.9	11				10
8	2	12	98.6	103.2	104.9	100.2	89.8		22	10	1	
9	2	12	99.1	103.3	104.2	99.7	89.6		25	0	4	
10	2	12	99.4	103.2	103.8	100.3	91.3		22	12	0	
11	2	24	99.6	101.7	103.0	99.5	—		22	8	1	
12	2	25	99.4	102.9	103.5	100.0	91.8	25				11
13	2	25	99.5	104.2	108.0	100.3	92.0	25				11
14	2	29+	98.6	103.1	104.1	99.0	88.4	29+				1
15	2	30+	99.2	102.6	103.8	100.0	90.0	30+				12
16	2	48	99.5	101.3	102.8	100.0	91.0		22	1	8	
17	3	3	99.0	103.8	104.7	99.8	91.3	3				8
18	3	4½	99.0	106.2	107.5	100.5	93.0	9+				9
19	3	5½+	98.8	102.8	102.8	100.0	90.0	5½+				11
20	3	5½+	99.2	103.3	103.3	100.0	90.0	5½+				10
21	3	5½+	99.0	101.6	102.0	99.8	91.3	5½+				11
22	3	7½	98.4	103.4	105.5	99.7	93.0	7½				11
23	3	12+	98.8	102.1	103.5	100.0	89.6	12+				8
24	3	24	98.8	101.2	102.4	99.7	90.5		22	10	0	
25	3	25+	98.8	101.8	103.2	99.4	92.3	25+				10
26	3	26	99.7	100.9	103.0	100.0	90.6		22	6	0	
27	3	26	98.8	101.2	103.8	99.7	90.7		20	11	0	
28	3	28+	99.0	101.8	103.0	99.2	92.0	28+				12

course of the experiment. A reservoir of normal saline provided a source for drinking. Wherever possible, rats from each trimester group were kept in the incubator for periods of twelve, twenty-four, and forty-eight hours, and their rectal temperatures were maintained within a range of 102 to 105 degrees Fahrenheit. An additional four nonpregnant rats similarly were subjected to fever of twenty-four hours' duration and sacrificed at periods less than twenty days afterward. Owing to the variability of heat tolerance demonstrated by individual animals, it was necessary to alter the incubator temperature during each period of fever induction. The relative humidity was maintained within a 64 to 79 per cent range.

In all cases resulting in delivery of viable litters, the young were sacrificed routinely at 1, 7, 21, 40, and 60 days of age, as the size of the litter allowed. The jaws then were prepared for histologic study by the double embedding technic.[8] Similar preparations were made from all mothers dying during the course of the experiment, as well as from their contained young, and from the four nonpregnant febrile rats.

FINDINGS

As seen in Table I, the later in pregnancy that pyrexia was induced, the greater was the incidence of maternal death. In the first trimester group, all animals survived the experimental procedure. It may be noted that but one rat of this series (No. 6) failed to complete a normal course of pregnancy. In light of vaginal bleeding at approximately eight hours after the onset of fever, it is probable that abortion had occurred. There was no subsequent progression of pregnancy and the animal was sacrificed on the twenty-sixth day after breeding. No products of conception were noted. In contrast to the first group, the mortality rate of the second and third trimester animals was 50 and 75 per cent, respectively. This high incidence of death from fever allowed correspondingly fewer successful pregnancies. With the exception of two of the five delivered mothers in the second trimester group, all animals surviving their course of pregnancy delivered either entirely viable litters or litters with but one dead young. Seemingly no correlation exists between the degree of pyrexia and the occurrence of stillbirths. Although possibly due to circumstance alone, it may be of some interest to note that the smallest litters, numbering four young each, were of mothers having the longest gestational periods. In the case of Rat 14 that died after twenty-nine hours of fever, no evidence of abortion was apparent to account for the single fetus found at autopsy. However, it is possible that such an event had escaped observation.

In no instances were gross dental abnormalities noted in any of the treated rats or their surviving offspring. However, definite changes were observed by histologic examination. These were manifested in the pyrexic maternal rats without apparent correlation with duration of fever. As seen in the photomicrographs, a prominent feature of this tissue response was a disturbance in amelogenesis. One such typical change is demonstrated in the maxillary left incisor of Rat 20 (Fig. 1). A number of large, irregular masses of enamel-like composition project inferiorly from the ameloblastic layer and

occupy a space between cells and surface of the organic enamel matrix. The overlying epithelial papillae and retroepithelial connective tissue are markedly hyperemic. Although the ameloblasts are somewhat disorganized in arrangement, they show no striking abnormalities. Further apically (Fig. 2), the inner enamel epithelium shows a complete loss of its usual architectural pattern. Replacing the cell layer is a widened zone of edematous appearance containing a number of large, amorphous, basophilic masses. No enamel matrix or dentin has been formed in this region of the developing tooth. Other significant abnormalities of the enamel organ are well demonstrated in the mandibular left incisor of one of the nonpregnant rats that was sacrificed twenty days after termination of fever (Fig. 3). Located in the ameloblastic zone are multiple, large, cystlike formations containing occasional pyknotic and karyolytic nuclei. Several of these vacuolated areas are seen to impinge upon the overlying epithelial papillae. The outer connective tissue shows some increased vascularity. A photomicrograph (Fig. 4) of another region of this tooth shows similar vacuolated or cystic areas in the zone of ameloblasts and epithelial papillae. Many of these are seen to contain a basophilic material which, upon close inspection, is found to have a typical enamel prism architecture arranged in a whirllike pattern. Much of the underlying enamel matrix is decalcified. However, numerous small, basophilic droplets are present immediately beneath the altered ameloblastic layer.

Probably denoting more advanced disturbances in the function of amelogenesis are regions of arrested enamel matrix formation. Such an abnormality is demonstrated in the mandibular left incisor of another of the nonpregnant febrile rats that died at the termination of twenty-four hours' fever (Fig. 5). The defect is bordered on either side by a layer of enamel matrix which is demarcated from the underlying enamel by an irregular, interrupted band of dark-staining, amorphous material. Clearly evident are the terminations of enamel rods of both enamel layers as they proximate or seemingly attach to the irregular band of enamel-like substance. On the basis of these histologic findings, it is probable that, after forming an apparently normal layer of organic enamel matrix, the ameloblasts were injured and deposited the amorphous layer. The degree of cellular damage in the mid-region of the photomicrograph was probably irreversible as evidenced by the complete arrest of amelogenesis. On the other hand, the presence of enamel on either side of the craterlike defect is indicative of a reversible cell injury in these areas. Since the maximum thickness of enamel matrix bordering the zone of hypoplasia is 17 to 25 microns, it is entirely possible to relate this occurrence to the twenty-four hours of life following induction of fever. Although the average approximate rate of appositional growth, as determined by Schour and Hoffman[9] is 16 microns per twenty-four hours, it may be that a slightly accelerated apposition was an accompaniment of the abnormal amelogenesis.

In contrast to the described changes in the enamel organ, the dentin and odontoblasts of the pyrexic rats were relatively free of abnormalities. However, pulpal hyperemia was a rather consistent finding.

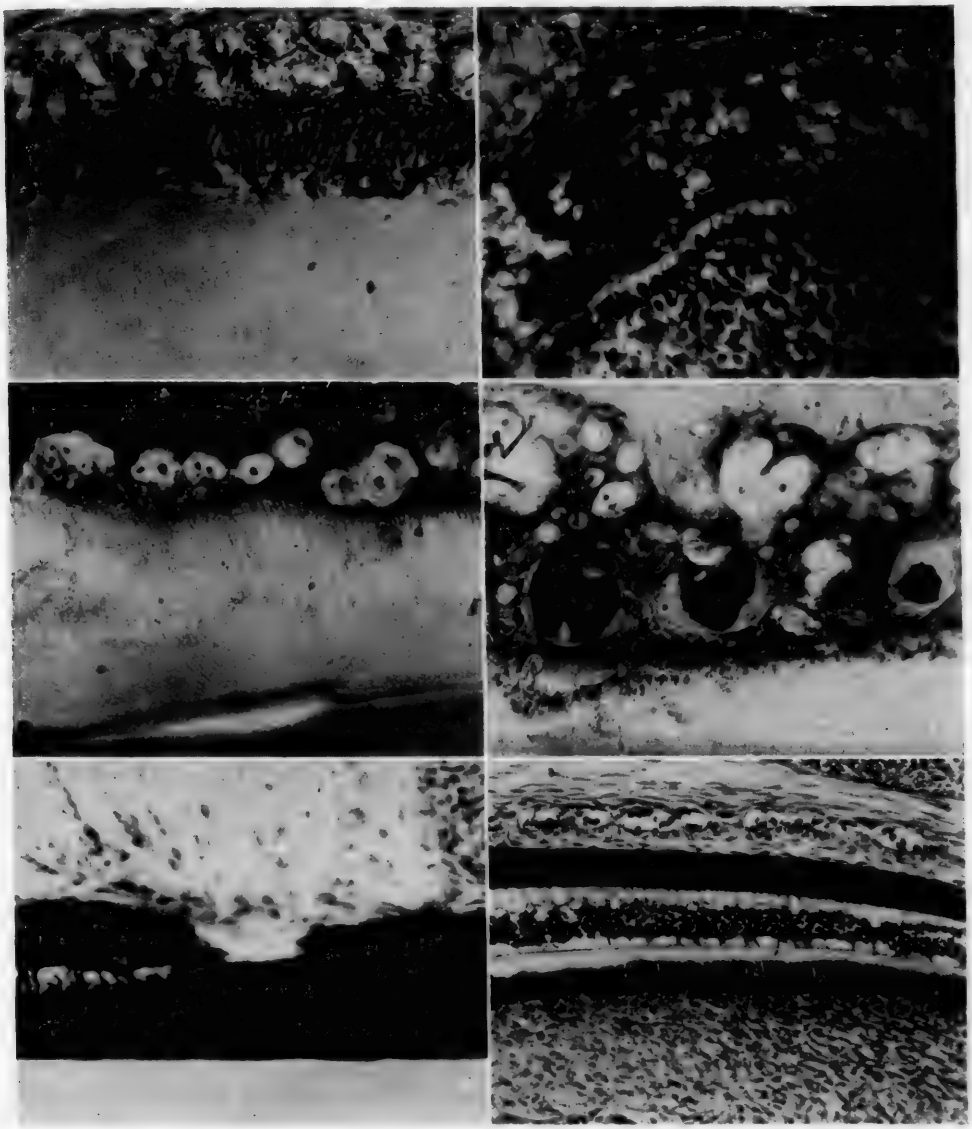

Fig. 1.—Maxillary left incisor of Rat 20 showing irregular, enamel-like formations between ameloblastic layer and surface of organic enamel matrix. (×250; reduced 2/5.)

Fig. 2.—Region apical to Fig. 1, showing degenerative and edematous-like changes in the inner enamel epithelium. Several large, deeply basophilic, amorphous masses are present in this area. (×400; reduced 2/5.)

Fig. 3.—Mandibular left incisor of nonpregnant febrile animal showing multiple, cystlike formations in ameloblastic zone. (×400; reduced 2/5.)

Fig. 4.—Region adjacent to that in Fig. 3. Large, ovoid formations of irregular enamel matrix are seen in region of ameloblasts and epithelial papillae. There is complete disappearance of organized cellular elements and many small, basophilic droplets overlie organic enamel matrix. (×400; reduced 2/5.)

Fig. 5.—Mandibular left incisor of another nonpregnant febrile rat showing area of arrested enamel formation. There is complete absence of ameloblasts in this region. (×400; reduced 2/5.)

Fig. 6.—Maxillary left incisor of a 40-day young of Rat 3. Separating ameloblasts from predentin is large accumulation of red blood cells. No enamel matrix has yet formed in this apical region. Ameloblasts and pulpal tissue are not unusual. (×200; reduced 2/5.)

With few exceptions, the offspring of the pyrexic animals showed abnormalities of enamel formation. These changes were noted in the incisor teeth as well as in those molars still undergoing amelogenesis. Other significant disturbances were observed in the odontoblasts and dentin. As noted in Table I, only thirteen of the febrile mothers terminated their pregnancies with full-term deliveries. Consequently, the number of viable young available for study was somewhat limited. It is of further interest that but four litters failed to show any dental abnormalities. These were all of mothers that had been rendered febrile prior to the ninth day of gestation (Rats 2, 5, 8, and 9). Of the remaining mothers that were pyrexic before the ninth day of pregnancy, the young of Rats 1 and 4 showed changes limited entirely to the odontoblasts, and the young of Rat 3 were negative except for the 40-day sacrifice. Since the siblings of this latter offspring, sacrificed at 1, 7, and 21 days of age, showed no dental abnormalities, it is likely that the finding noted in Fig. 6 was unrelated to the maternal fever. Furthermore, the abnormality, on the basis of its extreme apical position, is apparently of recent occurrence. As seen in the photomicrograph, a large number of red blood cells separate ameloblasts from dentin. In light of this unusual finding, consideration was given to the possibility of its representing artefact. However, persistence of this picture in serial sections through the entire block of tissue affords some confirmatory evidence of its pathologic nature.

The litters of the remaining seven delivered mothers showed abnormalities involving the enamel organ, as well as odontoblasts and dentin. With but one exception, namely, the litter of Rat 27 which showed changes in all sacrificed young up to 40 days, these findings were limited to those offspring sacrificed at 7 or less days of age. Typical of many of the examined sections is that seen in the maxillary incisor of a 7-day young of this animal (Fig. 7). Overlying the organic enamel matrix is an irregular, basophilic band of enamel-like material. Artefact separates this material from the ameloblasts which are of normal appearance. A small number of degenerated cells are found subjacent to the ameloblastic layer. Immediately beneath the abnormal

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Fig. 7.—Maxillary right incisor of a 7-day young of Rat 27 showing irregular layer of amorphous, enamel-like material on surface of organic enamel matrix. Ameloblasts show no apparent abnormalities. (×700; reduced 2/5.)

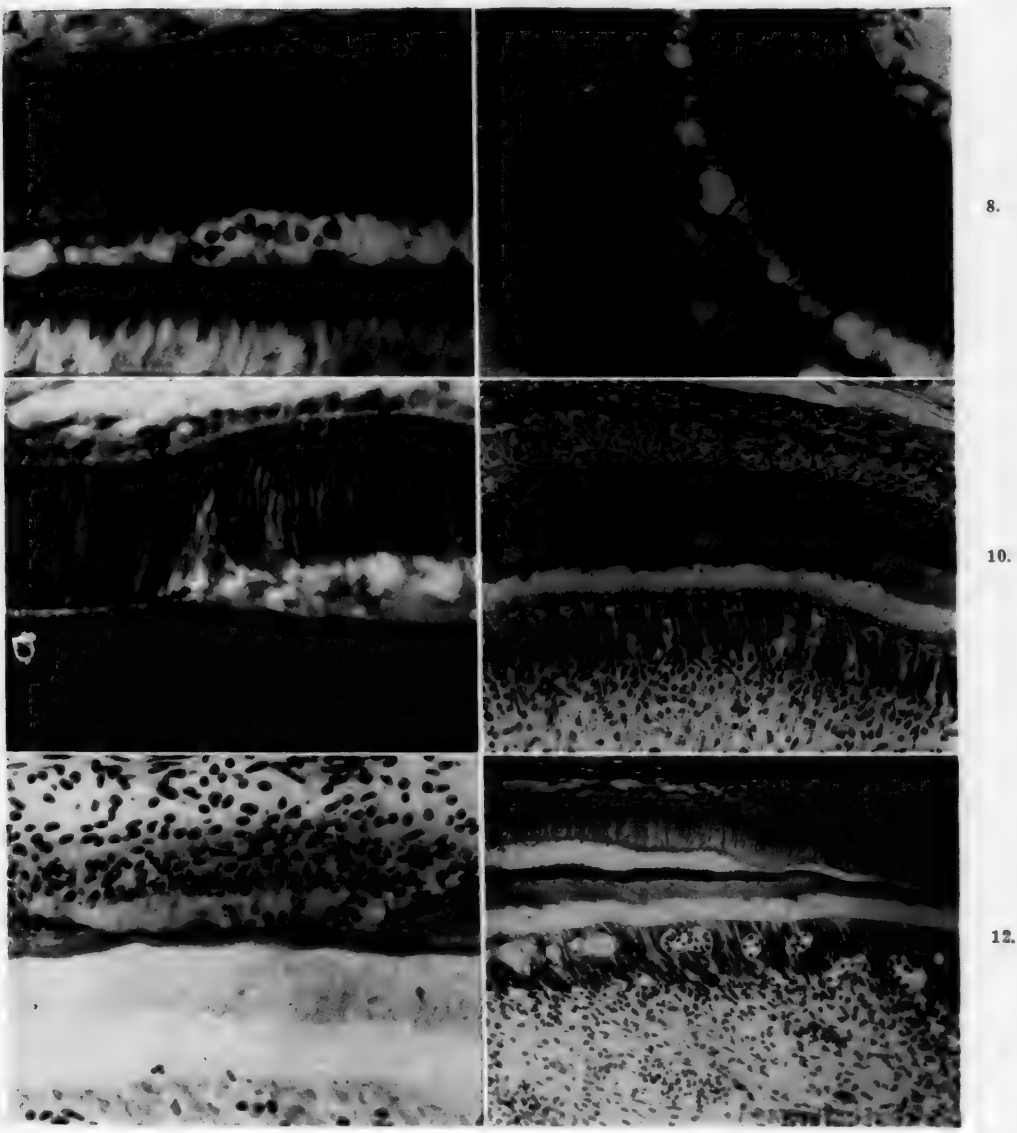
Fig. 8.—Maxillary right first molar of animal in Fig. 7. Separating ameloblasts from organic enamel matrix is large accumulation of edematous material containing numerous degenerated cellular elements. (×450; reduced 2/5.)

Fig. 9.—Another region of the molar in Fig. 8. There is abrupt loss of ameloblastic basement membrane in exudative zone and cells in mid-field show evidence of early degenerative change. (×400; reduced 2/5.)

Fig. 10.—Maxillary incisor of 1-day young of Rat 24. Ameloblasts at extreme right and left are intact in appearance, although they show progressive irregularity and diminution in size as they approach center of field. Underlying organic enamel matrix shows similar progressive decrease in thickness. Except for two prominent elevations, there is almost complete absence of enamel matrix in large zone of ameloblastic degeneration. Primary and predentin are of approximately equal thickness and odontoblasts show questionable degree of disorganization. (×200; reduced 2/5.)

Fig. 11.—Higher magnification of another region of the incisor in Fig. 10. There is extensive ameloblastic degeneration with arrest of enamel matrix formation and deposition of two deeply basophilic masses in center. To right and left of this zone, somewhat disorganized ameloblastic layer again makes an appearance and overlies moderately basophilic organic enamel matrix. As in previous illustration, there is widening of predentin zone. (×400; reduced 2/5.)

Fig. 12.—Maxillary incisor of 1-day young of Rat 16 showing increased vascularity of odontoblastic zone and wide predentin layer. Ameloblasts and enamel are of normal appearance. (×200; reduced 2/5.)



Figs. 7 to 12. (*For legends, see opposite page.*)

enamel substance can be seen the uniform, spikelike terminations of the enamel prisms. The remaining enamel matrix shows little staining reaction except for fiberlike projections from the terminal rods. Fig. 8 shows a somewhat similar finding in one of the molar teeth of the same animal. Occupying a zone between ameloblasts and enamel surface is an edematous-like material containing numerous vacuolated areas and degenerated cells. Neither the ameloblastic layer nor the organic enamel matrix are of abnormal appearance. Although the basement membrane of the inner enamel epithelium is seemingly intact in this photomicrograph, an adjacent field (Fig. 9) shows an abrupt termination at the point of junction between normal tissue and edema formation. This might suggest that the site of origin of the edematous material is the ameloblasts in mid-field which show evidence of early degenerative change.

Representing a more advanced type of change in the enamel organ is complete degeneration of ameloblasts and arrest of enamel formation. Fig. 10, of a maxillary incisor from a 1-day young of Rat 24, illustrates such a typical picture. To the extreme right and left of the photomicrograph, the ameloblasts show a gradual diminution in size and increasing irregularity of contour as they approach the center of the field. A regular and uniform organic enamel matrix underlies these cells. However, in the region of complete ameloblastic degeneration, the enamel layer becomes very thin except for two prominent basophilic masses. A higher magnification of another region of this tooth (Fig. 11) shows the area of cellular degeneration in greater detail. The only remnants of ameloblasts are a number of pyknotic and karyolytic nuclei which overlie enamel-like, globular masses similar to those seen in Fig. 10. Both sections show a wide predentin zone and an odontoblastic layer which, except for a questionable degree of irregularity, is of normal appearance.

Associated with the described abnormalities of amelogenesis were many instances of odontoblastic change and abnormal dentinogenesis. Fig. 12 shows a marked increase in vascularity of the odontoblastic layer and a wide predentin zone in the maxillary incisor of a 1-day young of Rat 16. Ameloblasts and enamel matrix are not unusual. A higher magnification of another region of this tooth shows the extent of the engorged vascular channels (Fig. 13). Although an appreciable vascularity is normally present in the odontoblastic zone, as evidenced by study of the litters of five nonfebrile control rats, the degree is considerably less than in the experimental group.

In several instances, a peculiarly abnormal zone was present between odontoblasts and dentin. Such a finding is illustrated in the maxillary molar of a 7-day offspring of Rat 1 (Fig. 14). Separating the odontoblasts from predentin is a large edematous-like zone containing many red blood cells. Although an initial impression is that capillary vessels are present in this area, close inspection shows the vacuolated spaces to have no endothelial structure. The predentin is well demarcated from primary dentin and is of uniform width in all regions of the tooth. Considerable difficulty is met in resolving the question of whether the described picture is due to artefact.

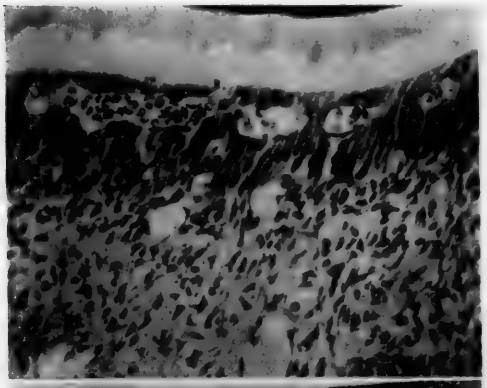


Fig. 13.

Fig. 14.

Fig. 15.

Fig. 13.—Higher magnification of area adjacent to that in Fig. 12. Extent of engorged vascular channels is clearly evident. (×400; reduced 2/5.)

Fig. 14.—Maxillary second molar of 7-day young of Rat 1 showing apparent detachment of odontoblastic layer from well-defined predentin, and accumulation of edematous-like material with numerous red blood cells in zone of separation. This may represent a poorly formed dentin matrix. (×400; reduced 2/5.)

Fig. 15.—Mandibular incisor of 7-day young of Rat 10 showing mottled appearance of dentin, probably denoting disturbance of calcification. (×400; reduced 2/5.)

In favor of this possibility is the absence of any evidence of arrested dentinogenesis in the zone of apparent detachment of odontoblasts from predentin border. Certainly, if this separation is due to edema and extravasation of blood cells and had been present for any appreciable period prior to animal sacrifice, some evidence of arrested development would be expected. On the other hand, the repeated demonstration of this finding in serial sections, as well as in a previous study of alloxan-injected rats, is suggestive of pathologic change. Furthermore, the possibility is considered that the edematous-like formation may merely represent an abnormally formed predentin matrix. A somewhat similar abnormality was reported also by Blackberg and Berke in their study of the young of vitamin D-deficient pregnant dogs.[10]

Further evidence of abnormal dentin formation is provided by the finding of an interglobular-like dentin in the young of the febrile rats. Such a picture is demonstrated in the mandibular incisor of a 7-day offspring of Rat 10 (Fig. 15). The predentin zone is sharply demarcated from the overlying dentinal tissue which shows a markedly mottled appearance, suggesting an abnormality of calcification rather than of matrix formation. Odontoblasts are essentially normal in appearance.

DISCUSSION

As proposed in the introductory remarks of this report, it is conceivable that the reason so wide a variety of disease processes elicit a similar dental tissue response is that the effect is mediated through certain common pathways or denominators of disease. On the other hand, it is possible that the high degree of differentiation of ameloblasts and odontoblasts would predispose them to a ready and similar tissue response to a variety of injurious agents. Although pyrexia is a common accompaniment of many diseases, the dental histopathologic findings in the present study do not necessarily provide a valid indication of its specific etiologic relationship. Similarly, any attempt to explain the high incidence of maternal death in those animals rendered febrile during the later stages of pregnancy would be conjectural. For example, it is entirely within the realm of possibility that fever per se was not the initiating factor in death, but rather served as a condition of stress which, when superimposed upon the already existing stress of pregnancy, was sufficient to initiate a terminal episode.

In light of the high degree of specialization of ameloblasts and odontoblasts, it is not surprising that the number of disease processes and other factors etiologically related to abnormal odontogenesis is being continually enlarged. One might be inclined, therefore, to attach a certain limited degree of value to such repeated demonstrations of cause and effect. However, the significance of these studies becomes apparent when one considers that dental tissues are unique in affording pictorial evidence of previous disease as well as serving as an unusually sensitive indicator of the generalized or systemic nature of disease processes.

Although a few studies have been made of the influence of fever on developing dental tissues,[4-6] no previous reports have been found which sought to determine whether pyrexia in the gestational animal also can affect the

oral structures of the young in utero. Also unlike previous studies which described changes only in the odontoblasts and dentin, the present report demonstrates changes in amelogenesis as well. Possibly this difference in findings is due, in part, to varied methods of tissue preparation. Inasmuch as Berman, Edwards, and Kitchin[5] apparently limited their report in rabbits to thin ground sections of the incisor teeth, an omission of ameloblastic observations is to be expected. However, since Garrison's study[4] of pyrexic rats included decalcified material, failure to mention the enamel organ might be assumed to indicate negative findings. On the other hand, Bevelander and Bernstein,[6] inducing fever in rats by short-wave emanations, reported changes specifically limited to pulp and dentin. As mentioned previously, jaw specimens for histologic study were removed from only those maternal rats dying during or shortly after pyrexia. The duration of fever varied from three to approximately thirty hours. Since it was desired that the young of delivered animals be studied at ages ranging from 1 to 60 days, the nursing mothers were spared from sacrifice. Although the resulting limitation of material for study is unfortunate in not permitting a longer post-febrile observation period, the examined rats afford a more certain etiologic correlation of dental changes and fever. Thus, should longer-range observations be made, some method such as alizarin injection followed by ground section preparations would be necessary in order to date cause and effect.

Of special interest in the febrile rats are the striking changes in amelogenesis. These findings show no significant correlation of severity with duration of fever or stage of pregnancy. As noted in previous studies, such abnormalities undoubtedly are nonspecific in nature. It is not surprising, therefore, that certain of the observed changes in this report bear a marked similarity to those described by other investigators and attributed to such varied factors as avitaminosis A and D,[11-15] magnesium deficiency,[16] hypoparathyroidism,[17] tuberculosis,[18] etc. Thus, it may be suggested that histopathologic changes in amelogenesis are not pathognomonic of specific etiologic factors. It is conjectural whether such a contention is applicable to macroscopically demonstrable dental defects. Certainly, on the basis of several of the cited reports as well as histologic observations made by Diamond and Weinmann,[19] Karnosh,[20] and Burket,[21] the rather frequent practice of designating teeth as grossly pathognomonic of rickets or syphilis may be viewed with some skepticism.

Of principal interest in the present study are developmental dental abnormalities noted in the young of mothers rendered pyrexic during pregnancy. To our knowledge, such findings have not been reported previously. As in the case of a previous investigation which demonstrated almost identical abnormalities in the young of alloxan diabetic rats,[1] we can only conjecture about the mechanism of injury to the formative dental structures. For example, an elevated fetal temperature occurring concomitantly with that induced in the maternal organism, may affect tooth development in the young just as it does in the mother. However, no information has been obtained regarding temperature of the young in utero.

An alternative possibility is that certain metabolic or endocrine processes of the mother may be disturbed, thereby initiating an effect on the fetus which is indirectly related to the maternal pyrexia. Nevertheless, regardless of the suspected mode of pathogenesis, it is necessary to continue to recognize the varied and complex nature of congenital abnormalities in general, and the role of not only genetic, metabolic, and hormonal factors, but of transplacental transfer of infection in maternal diseases of spirochetal and viral etiology.

Since the current series of studies (including the previous report on alloxan diabetes in pregnant rats) have dealt primarily with possible etiologic factors that are not generally considered to be limited by the selectivity of the placental barrier, it is perhaps not surprising that the fetal dental tissues showed a pathologic response. However, the possibility exists that even in instances of microbial maternal disease, the injurious effects on the fetus may be mediated via metabolic or endocrine pathways rather than direct placental transfer of infection.

SUMMARY AND CONCLUSIONS

Thirty-three virgin Wistar strain rats were mated with normal males of proved fertility. Following conception, as demonstrated by spermatozoa in vaginal smears, five of the animals were reserved as controls and the remaining twenty-eight were grouped according to stages of pregnancy and subjected to artificially induced fever by means of an incubator equipped with ventilation and humidity controls. Six rats were treated in this manner during their first trimester of pregnancy, ten during their second, and twelve during their third trimester. Wherever possible, the animals from each group were kept in the incubator for periods of twelve, twenty-four and forty-eight hours, and their rectal temperatures were maintained within a range of 102 to 105 degrees Fahrenheit. An additional four nonpregnant rats were similarly subjected to fever of twenty-four hours' duration.

In fifteen cases resulting in delivery of viable litters, the young were sacrificed at 1, 7, 21, 40, and 60 days of age, as the size of the litter allowed, and their jaws were prepared for histologic study by the double embedding technic. Similar preparations were made from the five control rats and their litters, from the four nonpregnant febrile rats, and from the thirteen pregnant animals (and their contained young) dying during the course of the experiment.

Striking changes in amelogenesis were noted in the febrile rats. These ranged from manifestations of ameloblastic dysfunction to complete cellular degeneration with arrest of enamel matrix formation. Although pulpal hyperemia was a rather consistent finding, no significant changes were noted in the dentin or odontoblasts.

Similar abnormalities in amelogenesis were noted in the young of the mothers rendered pyrexic during their course of pregnancy. Additional disturbances were found in the dentin and odontoblasts.

Although this report provides additional evidence of the influence of prenatal disturbances on fetal tooth development, we may merely conjecture

about the mechanism of injury to the formative dental structures. In light of similar findings in a previous study of alloxan diabetes induced during pregnancy, it may be suggested that the prenatal period has a far greater influence on fetal dental development than is generally suspected.

The authors wish to acknowledge the technical assistance provided by Miss Susan Kimbrough and Mrs. Polly Peters.

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A SYSTEM FOR RECORDING ORAL CONDITIONS AND DENTAL LESIONS IN THE LIVE SYRIAN HAMSTER

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THE development of a device permitting repeated examinations of the teeth and surrounding soft tissues of living rodents[1] necessitated the designing of charts adapted to the recording of data from successive examinations. The charts discussed here (Figs. 1 to 5) have been developed for use with the Syrian hamster and make provision for recording the progress of eruption and attrition of the dentition, of dental caries, of the formation of supra- and subgingival deposits, and for following the different phases of periodontal disease under laboratory conditions. The individual charts are most conveniently employed if joined in book form.

Because of the excellence and the wide usage of the diagrammatic representation of the teeth of the hamster developed by Keyes[2] for the scoring of the jaws of sacrificed animals, his method has been employed as a model in the present work.

METHOD OF RECORDING

The use of colored pencils, a different color for each examination, greatly facilitates the recording and differentiation of successive observations on the charts.

State of Eruption and Attrition.—It is often desirable to record the degree of eruption of the individual teeth prior to the institution of an experimental regimen. This is conveniently done on a chart identical to Keyes' caries chart[2] by drawing longitudinal lines across the buccal and lingual views of the teeth. Fig. 1 illustrates the system in use and shows the state of eruption of the teeth of the maxillary left quadrant of a 25-day-old hamster in which the third molars have not yet erupted. The eruption chart may also be employed when attrition is to be recorded; for this purpose a differently colored line is employed.

Dental Caries.—Several systems have been developed to record in a numerical evaluation the caries lesions in the Syrian hamster as evidenced in the stripped head of the sacrificed animal. The method reported by Keyes[2] has been widely used. To adapt the latter for recording dental caries in

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the live unanesthetized animal, two modifications have been introduced: (a) the buccal and lingual lesions are recorded in conjunction with the occlusal lesions, and (b) provision is made for the recording of interproximal lesions. Fig. 2 depicts the left one-half of the modified chart. Fig. 3 illustrates its practical application for three successive examinations. Because of these modifications the mesiolingual, mesiobuccal, distolingual, and distobuccal areas on all teeth will be relatively increased in size. All occlusal areas and the central buccal and lingual areas on the first molars will remain unchanged in representation, and the number of unit areas on the new tooth diagrams is the same as on Keyes' original chart. The criteria for estimation of cavity depth and the counting system are also retained.

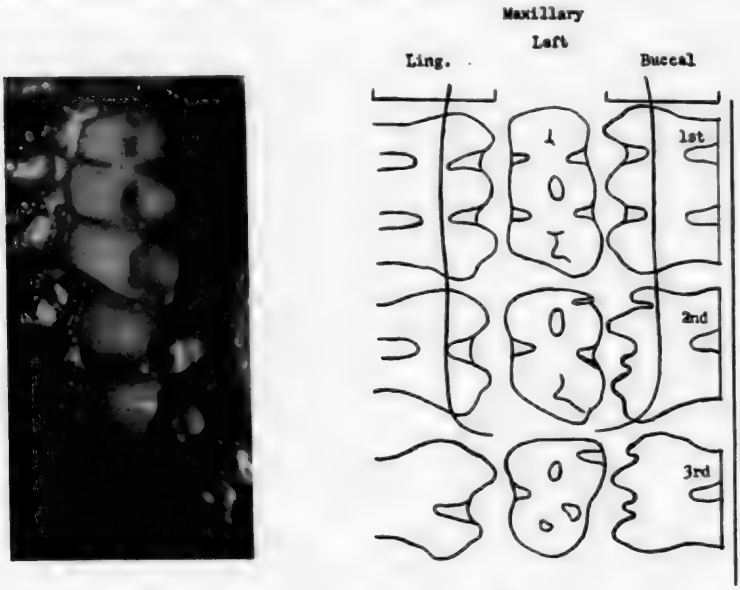

Fig. 1.—Eruption chart. The state of eruption of the molars is recorded by drawing longitudinal lines across the buccal and lingual diagrams of the teeth.

Since certain areas on the chart have been increased relatively in size, but since the Keyes' counting system is retained, there will of necessity be a slight difference in the resulting scores. When lesions are located interproximally, the scores on the new chart will be slightly larger than if recorded on Keyes' chart. On the other hand, if the lesion is located bucally or lingually the scores will be less. The difference in scores between the two methods will be of practical importance only when animals with minimal lesions are concerned.

Periodontal Disease.—A method for clinical estimation of periodontal disease in the sacrificed Syrian hamster has previously been reported by Mitchell.[3]

The chart depicted in Fig. 4 has been designed to record the lesion in the live animal. It allows a numerical evaluation of the lateral extent of periodontal pockets (gingival pushback), as illustrated in Fig. 5, and it may also be used to record the depth of the lesion.

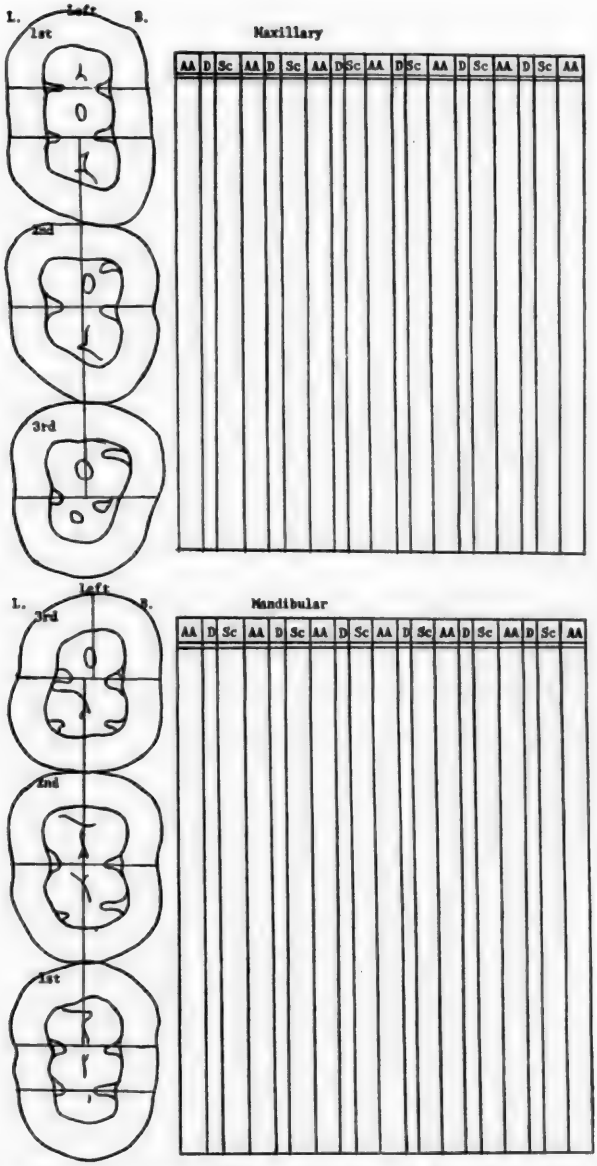

Fig. 2.—Dental caries chart. This modification of Keyes' chart for recording dental caries in the Syrian hamster makes provision for the recording of occlusal, buccal, lingual, and interproximal lesions on the same tooth diagram. The series of columns to the right of the diagrams provides space for data from successive examinations: *AA*, areas affected; *D*, depth; *Sc*, score.

Contrasting colors between the oral mucosa and foreign material filling the periodontal pocket make it easy to discern the lateral border of the lesion. The lateral extent is determined by comparing the width of the pocket with the average buccolingual diameter of the molars in the same quadrant. To facilitate the transfer of the dimensions to the chart, landmarks are created by drawing the outermost of the circumferential lines in each quadrant at a distance away from the teeth equaling the average buccolingual diameter of the molars. The operator thus is enabled to refer to the molar dentition as the standard reference. Measuring the lesion on an actual millimeter scale has proved too time consuming.

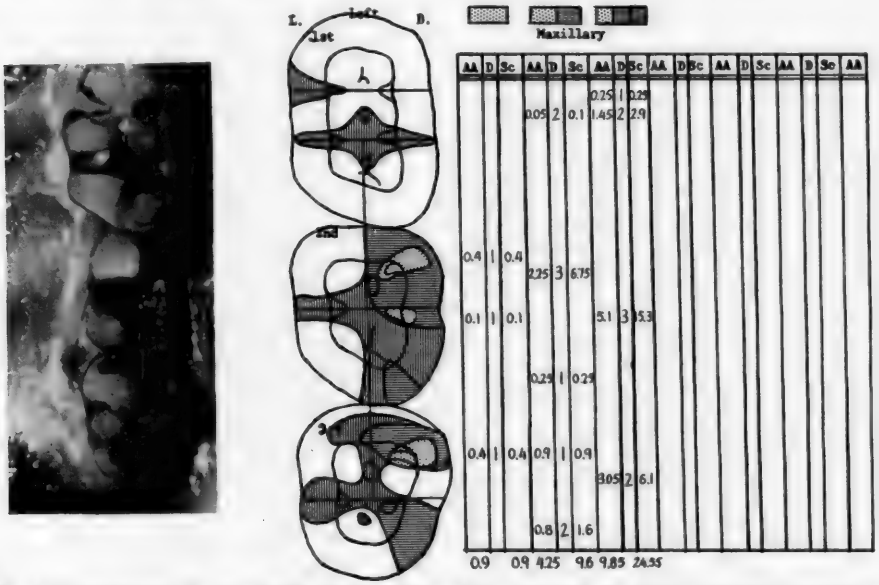

Fig. 3.—Application of the dental caries chart. The recording of three successive examinations with computations of data is illustrated.

Arbitrary units to facilitate the numerical evaluation of periodontal involvement have been represented by lines drawn in the following manner: first, the space between the outermost line and the teeth is divided into ten parts by lines nearly parallel to the circumferential line. Second, the perimeter of each tooth, excluding the interproximal surface, is divided into ten equal parts. By connecting the resulting points on the tooth surface with the peripheral line, areas roughly rectangular in form result. Each of these is considered one unit. The area between two radial lines is termed a section. Both designations are illustrated in Fig. 5, Quadrant *A*. By numbering each of the sections, and by specifying the quadrants as *A*, *B*, *C*, and *D*, the lesions can be located easily. The extent of the lesion is determined by the number of units involved.

Measuring the depth of the gingival sulcus and the pocket is an important part of any clinical study of periodontal disease. It is easily done in

the Syrian hamster by employing an explorer calibrated in millimeters. A chart identical to the one described for recording lateral extension of the pocket (Fig. 4) is used to record the depth. The distance between each of the circumferential lines is arbitrarily equated to 0.5 mm. in actual depth.

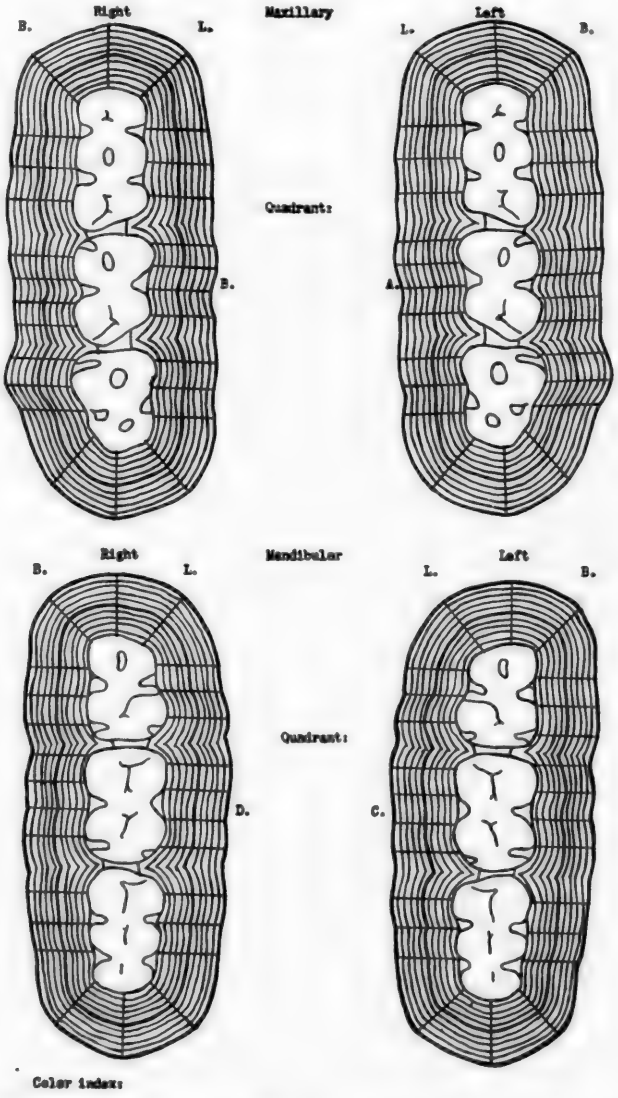

Fig. 4.—Periodontal chart. The gingiva is represented by lines encircling the tooth diagrams. Additional lines extending from the tooth surface to the peripheral line are drawn to produce units for the evaluation of the lateral extension of pockets. These lines have been omitted from the interproximal areas due to the anatomic configuration of the teeth. The interproximal section should therefore be regarded as representing two sections, doubling its number of units.

Lateral view diagrams similar to the ones used for recording eruption have been tried for this purpose, but were found to be less satisfactory than the periodontal chart which yields a clearer and more complete picture of the condition. Using identical charts for recording the lateral extent of the lesion and the pocket depth also facilitates a visual correlation between the two.

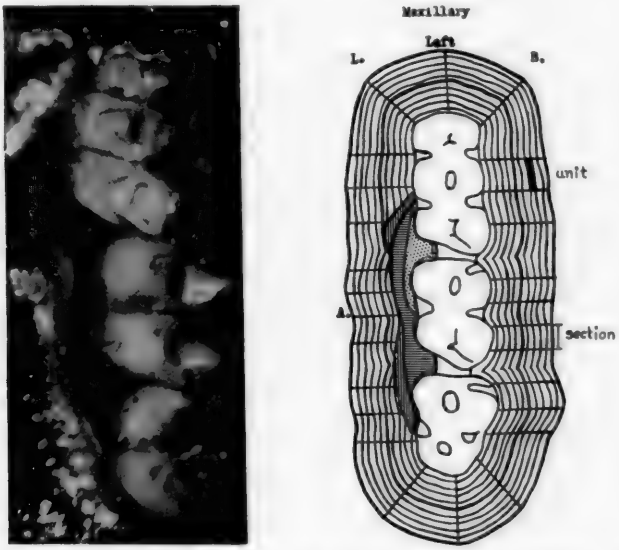

Fig. 5.—Application of periodontal chart. The recording of three successive examinations is illustrated. Key: same as on Fig. 3.

In certain studies, particularly of the lower first molar, it is also interesting to note the rate of recession of the gingival attachment. For this purpose the lateral view representation of the teeth on the eruption chart (Fig. 1) is useful. The rate of recession is recorded by drawing lines supplementing those already representing the state of eruption.

Supra- and Subgingival Deposits.—To study the relation between periodontal disease, dental caries, food deposits, and gingival impactions, special charts have been designed by combining the three types illustrated in this article.

Recording of tooth surface deposits and supragingival deposits can be done on a chart where the tooth diagram of the caries chart is surrounded by a gingival diagram similar to the one represented on the periodontal chart. The latter is added for the simultaneous recording of the lateral extent of the pocket and the supragingival deposits, which makes for an easy correlation between the two.

Subgingival deposits and food impactions can be recorded on a modified eruption chart in which the occlusal view representations have been replaced

by the periodontal chart diagrams. The substitution is carried out to permit a convenient correlation between gingival pushback and subgingival deposits, which is recorded on the lateral view diagram.

SUMMARY

A series of charts for the progressive recording of eruption and attrition of the dentition, of dental caries, and of the different phases of periodontal disease has been described and depicted. Composites of these charts for recording the accumulation of supra- and subgingival deposits have been discussed.

The author wishes to thank Dr. John W. Hein for his many helpful suggestions in designing the charts. The able assistance of Dr. D. Afonsky in preparing the photographic material is also acknowledged.

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THE AMINO ACID COMPOSITION OF ENAMEL PROTEIN

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BY THE use of paper chromatography, Block, Horwitt, and Bolling[1] showed qualitatively that human enamel eukeratin contained most of the known amino acids, and gave quantitative values for arginine, histidine, and lysine. Losee and Hess,[2] who had prepared the protein analyzed by Block, Horwitt, and Bolling, determined cystine, methionine, and phenylalanine by colorimetric methods and subsequently[3] also determined arginine, histidine, and lysine, and confirmed the statement that the normal enamel protein is an eukeratin. However, the sum of the values for these six amino acids accounts for less than 15 per cent of the total nitrogen of the protein. In order to characterize completely the protein and also to permit comparison with dentinal protein and other eukeratins, we have estimated the other amino acids in normal enamel protein.

EXPERIMENTAL

The enamel protein was prepared as described by Losee and Hess.[2] The nitrogen content was 13.59 per cent and the ash content was 9.59 per cent, based upon the weight of the desiccator-dried sample. The amino acids, except the six previously determined colorimetrically, were all estimated by the microbiologic methods used by Horn, Jones, and Blum.[4] The inoculums were prepared using the media of Hac, Snell, and Williams[5] because the liver infusion used by Horn, Jones, and Blum[4] was unavailable. *Leuconostoc mesenteroides* was employed for the assay of leucine and isoleucine, and *Streptococcus faecalis* for threonine and valine as recommended by Horn, Jones, and Blum.[4] Leucine, isoleucine, valine, glutamic acid, aspartic acid, proline, glycine, and tyrosine were determined, as suggested by Neuman,[6] using *Leuconostoc mesenteroides* and serine employing *Streptococcus faecalis*. For each amino acid from three to four concentration ranges and triplicate determinations for each concentration were made, of the resulting lactic acid using 0.05 N NaOH and bromthymol blue as the indicator. The results were averaged.

The protein was hydrolyzed with 20 per cent hydrochloric acid for six hours at 125° C., and the hydrolysate was freed from excess acid by evaporation under reduced pressure. The results of these analyses and the values for the six amino acids previously determined are given in Table I, together with the range of values for the amino acids in other eukeratins as cited by Block and Bolling.[7] The nitrogen content of the amino acids determined accounts for 98.3 per cent of the total nitrogen of the protein.

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TABLE I

AMINO ACIDS IN HUMAN ENAMEL PROTEIN

AMINO ACIDS	PER CENT	NITROGEN PER CENT	AMINO ACID BASED UPON 16 PER CENT NITROGEN PER CENT	LITERATURE VALUES[7] PER CENT
L-Lysine	3.75	0.718	4.41	1.1 – 4.5
L-Arginine	6.72	2.161	7.91	6.5 –10.7
L-Histidine	1.15	0.311	1.35	0.4 – 2.6
L-Phenylalanine	6.20	0.526	7.29	1.3 – 7.6
L-Methionine	1.01	0.095	1.18	0.5 – 5.4
L-Cystine	0.60	0.070	0.70	5.0 –20.0
L-Tryptophane	0.10	0.014	0.12	0.0 – 2.7
L-Serine	6.50	0.866	7.65	6.0 –10.2
L-Threonine	4.38	0.515	5.15	3.9 – 8.2
Glycine	18.75	3.499	22.06	4.5 –10.8
L-Alanine	8.12	1.276	9.55	1.0 – 4.0
L-Tyrosine	1.04	0.080	1.22	3.0 –13.0
L-Proline	4.43	0.340	5.21	8.5 – 9.3
L-Aspartic acid	5.00	0.526	5.88	7.9 –10.2
L-Glutamic acid	9.37	0.892	11.02	9.7 –17.8
L-Valine	3.25	0.388	3.82	2.7 – 8.9
L-Leucine	4.50	0.480	5.29	8.5 – 9.5
L-Isoleucine	1.75	0.187	2.06	3.6 – 6.4
L-Hydroxyproline	5 09	0.352	5.99	
Total	88.44	13.364	104.01	
		13.59 Found		
Ash content of enamel protein		9.59 per cent		

In order to compare the amounts of the amino acids in the enamel eukeratin with those in other eukeratins, the percentages have been converted to a 16 per cent nitrogen basis as recommended by Block and Bolling,[7] and the range of values of the same amino acids in other eukeratins as cited by them has been included in Table I. It will be noted that for all of the amino acids the ranges are quite broad. However, there are some conspicuous differences in composition between enamel and other eukeratins. The cystine and tryosine values of enamel eukeratin are less, while the glycine and alanine values are much higher than those in eukeratins in general. The values for the latter two amino acids resemble those found in dentinal protein.[8] However, the criterion that an eukeratin should contain the three basic amino acids, histidine, lysine, and arginine, in the molecular ratios of 1:4:12 is met by enamel eukeratin, but not by the collagen in dentinal protein. Glycine and alanine account for 36 per cent of the nitrogen in enamel eukeratin and 41 per cent of the nitrogen in dentinal protein; the latter protein contains 13 per cent hydroxyproline and 1 per cent hydroxylysine, whereas enamel eukeratin lacks hydroxylysine completely. Until we possess a greater knowledge of the biologic synthesis of proteins it would be fruitless to speculate on the possibility that dentinal protein serves as a matrix for the formation of enamel eukeratin.

SUMMARY

Of the twenty known amino acids, all except hydroxyproline and hydroxylysine have been found in normal human enamel eukeratin. The nineteen

amino acids have been determined quantitatively, and their nitrogen content accounts for 97.9 per cent of the total nitrogen of the protein.

ADDENDUM

Dr. M. Stack of Guy's Hospital has recently informed us that he has also found hydroxyproline in human enamel protein by paper chromatography and also by the method of Neuman and Logan,[9] which we employed. This amino acid, for which satisfactory analytical methods previously have not been available, has not been reported present in eukeratins.

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INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
1954 MEETING

The 32nd General Meeting of the International Association for Dental Research will be held at French Lick, Ind., March 19, 20, and 21, 1954.

The closing date for acceptance of papers for the program will be Dec. 15, 1953, and each request for program time should be mailed to Dr. Edw. H. Hatton, 311 E. Chicago Ave., Chicago, Ill. The Novice Award will be available again this year. Details are available from Dr. Hatton.